EFFECTS OF BOVINE COLOSTRUM ON IMMUNE RESPONSES TO PROLONGED EXERCISE AND UPPER RESPIRATORY ILLNESS IN ACTIVE MALES

A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy of Aberystwyth University

by Arwel W. Jones (2013)

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General Abstract

It is now well established that exercise of a strenuous and/or prolonged nature can lead to transient perturbations in immune function. The clinical significance of participating in such acute bouts of exercise in combination with other life stressors (e.g. inadequate nutrition) may be an increased incidence of upper respiratory illness (URI) (e.g. sore throat, runny nose). Many proposed nutritional countermeasures to exercise-induced immune dysfunction have been shown to be ineffective. The aims of this thesis were to determine the effects of bovine colostrum (COL) on *in vitro* and *in vivo* measures of immunity taken at rest and/or following prolonged (≥ 2 h) exercise and the incidence of URI during regular training in active males. Study 1 (Chapter 3) demonstrated that acute COL supplementation improved the recovery of bacterial stimulated neutrophil degranulation and enhanced salivary lysozyme concentration following 2.5 h of cycling. There was also greater fMLP-stimulated oxidative burst throughout the COL trial compared to PLA. These effects are suggested to be partly due to components and/or metabolites of COL that become bioavailable following digestion of the supplement which may explain why Study 2 (Chapter 4) demonstrated a greater effect on fMLP-stimulated oxidative burst with 4 weeks of COL supplementation. Study 3 (Chapter 5) found a lower proportion of URI days and lower number of URI episodes with 12 weeks of COL supplementation. Although there was no effect on selected measures of *in vitro* immune function taken at rest (fMLP oxidative burst, salivary secretory IgA and antimicrobial peptides), COL did blunt increases in salivary bacterial load over the winter period, which may provide a novel marker of *in vivo* immunity. Despite no effect of prior infection with Epstein-Barr virus (EBV) on URI incidence in Study 4 (Chapter 6), those who were seropositive and undergoing COL supplementation had a lower number of URI days

than seronegative counterparts. Study 5 (Chapter 7) observed a lack of effect of COL supplementation on the overall magnitude of an *in vivo* measure of T-cell-mediated immunity to a novel antigen following prolonged exercise but there was evidence that COL may increase the sensitivity of responses to such antigenic challenge.

Keywords: neutrophil function, mucosal immunity, URTI, Epstein Barr virus, DPCP

Acknowledgements

The past 41 months have been the most challenging but rewarding period of my life. Many people have assisted and supported me throughout the completion of this thesis and I would like to take the opportunity to mention these people in this section.

First and foremost I would like to acknowledge the Knowledge Economy Skills Scholarship scheme which via the Welsh Government has administered and partly funded (European Social Fund) a collaborative project with The Golden Dairy Ltd. My thanks go to John Rolfs (The Golden Dairy Ltd) for his efforts as a company supervisor in this project and his in-kind contributions of bovine colostrum powder throughout the duration of the project via Neovite UK.

A huge gratitude must go to my main supervisor Dr Glen Davison and co-supervisor, Dr Rhys Thatcher. Both have always been keen and willing to make time for me. Glen's attention to detail and incredibly vast knowledge and expertise in exercise immunology, nutrition and physiology was instrumental towards completion of the thesis and the opportunity to work with him was a motivating factor for me to take up the challenge of a PhD. Rhys's forthcoming advice and guidance have been invaluable towards management and completion of this thesis.

I would to like to thank Simon Cameron, a fellow PhD student at Aberstywyth for his hard work completing the measurements of salivary bacterial load, microbial growth and metabolomics over in the laboratories at the Institute of Biological, Environmental and Rural Sciences (IBERS) (Chapter 5 and Appendix B). It has been a pleasure working with him and his mentor Prof Luis Mur. It has also proved to be a successful collaboration that I intend to pursue with in the future. Credit must also go to IBERS Undergraduates who worked alongside Simon and submitted incomplete

datasets as their BSc dissertation. I would like to thank Krzysztof Nalborski, an Undergraduate of the Department of Sport and Exercise Science who as part of a work experience module on the BSc degree scheme assisted me in the running of main experimental trials of Chapter 4 and 7 by collecting expired gas samples, monitoring and recording heart rate and rating of perceived exertion. Any acknowledgements section would not be complete without thanks to all of the participants who volunteered for the experimental studies as this work would not have been possible without them.

Thanks to all fellow postgraduate research students and staff at the Department of Sport and Exercise Science for their friendship and making my time as a PhD student an enjoyable one. A special mention must go to two postgraduates who started the same time as me and who I shared an office with for the duration of my time as a PhD student, Daniel March and Ffion Curtis. Thanks for the peer support and constant office banter. I have thoroughly enjoyed our journey together.

I must acknowledge the role of my family, in particular my brother, father and mother who have always been there to support me during my progress through University. Last, but certainly not least, I would like to mention two very important people in my life, my partner Mari and the little man Tyler James who arrived in this world near the end of my first year as a PhD student. I will be forever indebted to the both of them for their understanding and tolerance of me frequently working unsociable hours or being away from home as a result of my research. There are not enough words that can express my gratitude and appreciation for all that Mari has done for me during the busiest periods of my PhD and just so I could focus on completing the thesis. Diolch yn fawr.

Publications

To date the following publications have been generated from the experimental work of this thesis:

Journal articles:

Study 3 (Chapter 5):

Jones, A. W., Cameron, S. J. S., Thatcher, R., Beecroft, M. S., Mur. L. A. J. and Davison, G. (2013). Effects of bovine colostrum supplementation on upper respiratory illness in active males. *Brain Behavior and Immunity*, doi: 10.1016/j.bbi.2013.10.032.

Conference contributions:

Study 1 (Chapter 3):

Jones, A. W. (2012). Immunonutrition for sport. *Science and the Assembly meeting of the Royal Society of Chemistry*, Cardiff, May 2012.

Jones, A. W., Thatcher, R. and Davison, G. (2011). The effect of acute bovine colostrum supplementation on neutrophil responses to prolonged cycling. *Annual Congress of the European Congress of Sport Science*, Liverpool, UK, July 2011.

Jones, A. W., Thatcher, R. and Davison, G. (2011). The effect of acute bovine colostrum supplementation on neutrophil responses to prolonged cycling. *British Association of Sport and Exercise Science Student Conference*. Chester, UK, April 2011.

Jones, A. W., Thatcher, R. and Davison, G. (2011). The effect of acute bovine colostrum supplementation on neutrophil responses to prolonged cycling. *International Society of Exercise and Immunology Symposium*, Oxford, UK, July 2011.

Study 3 (Chapter 5):

Jones, A. W., Cameron, S. J. S., Thatcher, R., Beecroft, M. S., Mur. L. A. J. and Davison, G. (2013). Exploring the mechanisms behind the effects of chronic bovine colostrum supplementation on risk of upper respiratory tract infection. *International Society of Exercise and Immunology Symposium*, Newcastle, Australia, September 2013.

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List of abbreviations

AA	arachidonic acid
AMPs	antimicrobial peptides
ANOVA	analysis of variance
APC	antigen presenting cell
BALT	bronchus-associated lymphoid tissue
BM	body mass
Ca^{2+}	calcium ion
CD	cluster of differentiation
CD4+	T helper cell
CD8+	T cytotoxic cell
CMV	cytomegalovirus
CL	chemiluminescence
CMIS	common mucosal immune system
CO_2	carbon dioxide
COL	bovine colostrum
COL^+	bovine colostrum group seropositive for Epstein Barr virus
COL^-	bovine colostrum group seronegative for Epstein-Barr virus
CRP	c-reactive protein
CV	coefficient of variation
d	deci
DAG	diacylglycerol
DI	direct injection
DI-ESI-MS	direct injection electrospray ionisation mass-spectrometry
DNA	deoxyribonucleic acid
DPCP	diphenylcyclopropenone

DTH	delayed type hypersensitivity
EBV	Epstein-Barr virus
EBV+/-	Epstein Barr virus seropositive/seronegative
f	femto
f	forward
fMLP	formyl methionyl leucyl phenylalanine
g	gram
g	gravitational acceleration
GALT	gut associated lymphoid tissue
G-CSF	granulocyte -colony stimulating factor
GET	gas exchange threshold
GH	growth hormone
GI	gastrointestinal
GM-CSF	granulocyte macrophage-colony stimulating factor
h	hour(s)
H_2O	water
HBSS	hank’s balanced salt solution
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance liquid chromotography
HR	heart rate
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IPAQ	international physical activity questionnaire
K3EDTA	ethylenediaminetetraacetic acid tripotassium
kcal	kilocalorie
kDa	kilodalton

kg	kilogram
km	kilometre
L	litre
LPS	lipopolysaccharide
m	metre
M	molar
MALT	mucosa associated lymphoid tissue
MET	metabolic equivalent
min	minutes
mg	milligram
MHC	major histocompatibility complex
mL	millilitre
mm	millimetre
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
n	nano
NADPH	nicotinamide adenine dinucleotide phosphate
NALT	nasal associated lymphoid tissue
NETs	neutrophil extracellular traps
NK	natural killer
NKCA	natural killer cell cytotoxic activity
O_2^-	superoxide ion
PA	phosphatidic acid
PBMC	peripheral blood mononuclear cells
PCA	principal component analysis
PCR	polymerase chain reaction

phox	phagocyte oxidase
pIgR	polymeric immunoglobulin receptor
PIP2	phosphatidylinositol (4,5) biphosphate
PIP3	phosphatidylinositol (3,4,5) triphosphate
PKC	protein kinase C
PLA	placebo
PLA2	phospholipase A2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol-12-myristate-13-acetate
PMN	polymorphonuclear
PP	Peyer's patches
PRR	pattern recognition receptor
qPCR	quantitative polymerase chain reaction
r	reverse
RLU	relative light units
ROS	reactive oxygen species (free radical)
RPE	rating of perceived exertion
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
s	seconds
SIgA	secretory immunoglobulin A
sLac	salivary lactoferrin
sLys	salivary lysozyme
T^c	T cytotoxic cell
TGF	transforming growth factor

T^h	T helper cell
Th	Thomson
TLR	toll-like receptor
TNF	tumour necrosis factor
T^{reg}	T suppressor/regulatory cell
T-RFLP	terminal restriction fragment length polymorphisms
URI	upper respiratory illness
URS	upper respiratory symptoms
URTI	upper respiratory tract infection
VCA	viral capsid antigen
$\dot{V}O_2$	oxygen uptake
$\dot{V}O_2$ max	maximum oxygen uptake
W	watts
α	alpha
β	beta
Δ	delta
γ	gamma
μ	micro
°C	degrees celsius

Chapter 1. Literature Review

1.1 Exercise and upper respiratory illness

The majority of visits to general practitioners worldwide are due to middle ear infections, common cold, coughs and throat infections, collectively termed upper respiratory tract infections (URTI) (Eccles, 2005; Graham, 1990; Gulliford et al., 2009). Given that the ability of the immune system to resist infection is affected by environmental, psychological and physiological stressors (e.g. exercise), URTI are also suggested to be the most common type of infection in the athletic population (Cannon, 1993; Gleeson and Walsh, 2012; Peters, 1997, Roberts, 1986). In fact they have shown to be the most prevalent medical condition within athletes at clinics in both the summer and winter Olympic Games (Derman et al., 2013; Engebretsen et al., 2010, 2013; Robinson and Milne; 2002).

As maintenance of health and performance are the primary objectives of athletes (Matthews et al., 2010), there is a demand for an improvement in the prevention, diagnosis and treatment of these illnesses within the athletic population (Spence et al., 2007). Athletes have been reported to suffer from a similar rate of URTI to the general population (Fricker et al., 2000). However, as these episodes of URTI do not always follow the usual pattern of winter prevalence of URTI in the general population, it may reflect a mediatory role of exercise habits (Cunniffe et al., 2011; Matthews et al., 2010; Pyne et al., 2001). Although patterns vary between sports, a review by Walsh et al. (2011b) suggested that athletes tend to report URTI either during the high intensity and tapering period prior to competition (e.g. swimming, team sports) or in the period following competition (e.g. long distance running).

It has long been hypothesised that a J-shaped relationship exists between exercise workload and susceptibility to URTI (Figure 1.1A) (Nieman, 1994). This model suggests that an individual involved in regular moderate exercise is less likely to contract URTI compared to a sedentary individual but prolonged high-intensity exercise or periods of strenuous exercise training are associated with an above-average risk of URTI. Indeed, the J-shaped model was initially based on findings of increased self-reporting of URTI in the 1-2 week period following participation in competitive endurance races (e.g. Nieman et al., 1990a).

Further support for an adverse effect of prolonged/strenuous exercise on susceptibility to URTI has come from animal studies (Davis et al., 1997; Folsom et al., 2001; Gross et al., 1998; Lowder et al., 2005; Murphy et al., 2008). Prolonged exercise (treadmill running for > 2 h) has been shown to increase morbidity and mortality of mice inoculated with respiratory viruses (e.g. herpes simplex type 1 virus, influenza) prior to (Davis et al., 1997; Murphy et al., 2008) or following (Lowder et al., 2005) this type of exertion compared to resting and/or moderately exercise mice. In addition to acute exertion, equine studies have also demonstrated that intensified periods (5 - 28 days) of exercise training prior to or following inoculation with influenza leads to greater severity of infection in vaccinated (Folsom et al., 2001) and non-vaccinated horses (Gross et al., 1998). Generalising these results to the human *in vivo* environment is questionable (Albers et al., 2005) and such approaches (i.e. pathogen challenge) with human volunteers do have considerable ethical constraints (Hope and McMillan, 2004). Although not yet investigated in an exercise context, pathogen challenge studies in humans have demonstrated lower resistance to URTI due to other life stressors (e.g. psychological stress, sleep disturbance) (Cohen et al., 1991, 2009). In addition to the potential exposure to these stressors in a training

and competition environment (Coutts et al., 2007; Hausswirth et al., 2013), the relevance of these findings to athletes is emphasised by the impact of transient modulation in host resistance to URTI by a stressor.

Exercise immunology research (epidemiological and experimental) has grown substantially over the last few decades to investigate the relationship between exercise and URTI in humans (Shepard, 2010). In contrast to animal research, human studies (attempting to discern the effects of prolonged exercise/intense training on URTI) have mainly involved monitoring athletes following heavy exertion (i.e. relied on natural exposure to pathogens) but only a limited number of these have verified that symptoms are due to infectious agents (pathogens) (Schwellnus et al., 2010). This has raised concerns regarding the validity of URTI episodes (i.e. self-reported) in athletes that occur in and around competition or heavy periods of training (Bermon, 2007; Walsh et al., 2011b). Discrepancies between physician and laboratory diagnosed URTI has also highlighted the limitations with evaluation of URTI episodes (Cox et al., 2008). Upon presentation of symptoms at a sports medicine clinic, the study of Cox et al. (2008) found that only 57% of cases were found to be indicative of infection with laboratory methods (e.g. identified pathogen) while 89% were diagnosed as URTI by physicians. In a surveillance study of a range of athletes (recreational and elite) and sedentary controls, Spence et al. (2007) demonstrated that the first 2 days of symptoms with infectious or non- infectious (see later section 1.2) cases were similar but duration and severity of symptoms on subsequent days were greater with infectious cases. It is for these reasons that unless studies have confirmed (i.e. performed serological analysis and/or pathogen identification) that upper respiratory tract symptoms are due to an infection, episodes will be referred to as upper respiratory illness (URI) for the remainder of this thesis.

Although there were numerous early anecdotal reports and retrospective survey data to support the proposed J-shaped relationship (Nieman, 2000; Shepard et al., 1995; Simon, 1987), such observations alone cannot validate the influence of exercise on URI. However, further support to the J-shaped model (i.e. heavy exercise workload) was provided by a number of prospective and retrospective studies which suggested that marathon or ultramarathon runners were examples of athletes who may suffer from an increased risk of URI (e.g.1-2 weeks following competitions) (Nieman et al., 1990a; Peters, 1993). Peters and Bateman (1983) were among the first key studies to highlight this by randomly recruiting a sample of 140 runners who competed in the 1982 Two Oceans Marathon in Cape Town. In the 14 days following the 56 km event, 33% of runners reported URI compared to 15% of age-matched controls that did not participate in the marathon but shared living space with runners (i.e. control for exposure to pathogens).

Compared to equally experienced runners who did not compete, there was a six fold increase of URI in runners during the 7 days following the 1987 Los Angeles Marathon (Nieman et al., 1990a). Taking into account other factors influencing risk of URI (age, stress levels and illness at home), the likelihood of URI was doubled in those who ran > 96 km compared to those who ran < 32 km as part of their weekly training programmes leading up to the event. Heath et al. (1991) also highlighted running mileage as a significant risk factor for incidence of URI in a cohort of runners followed for a period of 12 months. More recently, Matthews et al. (2010) have also suggested that runners with higher training loads tend to be more prone to URI and that endurance athletes in particular suffer from longer episodes of URI than their recreational counterparts. However, such findings have not been demonstrated

consistently as shorter observational studies have failed to observe any associations in URI with differences in training mileage, intensity and load (Fricker et al., 2005).

Despite much interest, there remain to be more uncertainties than evidence based facts regarding the notion that high volumes of training are associated with an increase in the incidence of URI (Walsh et al., 2011b). Such inconsistent findings may be related to whether participants within studies are considered ‘elite’ or ‘highly trained’. Malm (2006) suggests that elite athlete status possesses a prerequisite for an immune system which can withstand the strenuous nature of training and competition as susceptibility to infections is incompatible with elite performance. For this reason, Malm (2006) proposed an S-shaped rather than a J-shaped curve to include elite training which is associated with a lower risk of infection compared to high exercise workload (Figure 1.1B). However, it is worthy to note that this may not reflect a reduced physical stress (exercise workload) *per se* but rather a reflection of the preventive and treatment strategies in place within elite settings to limit the effects of URI (i.e. maintain a pre-requisite of an elite athlete to withstand infections).

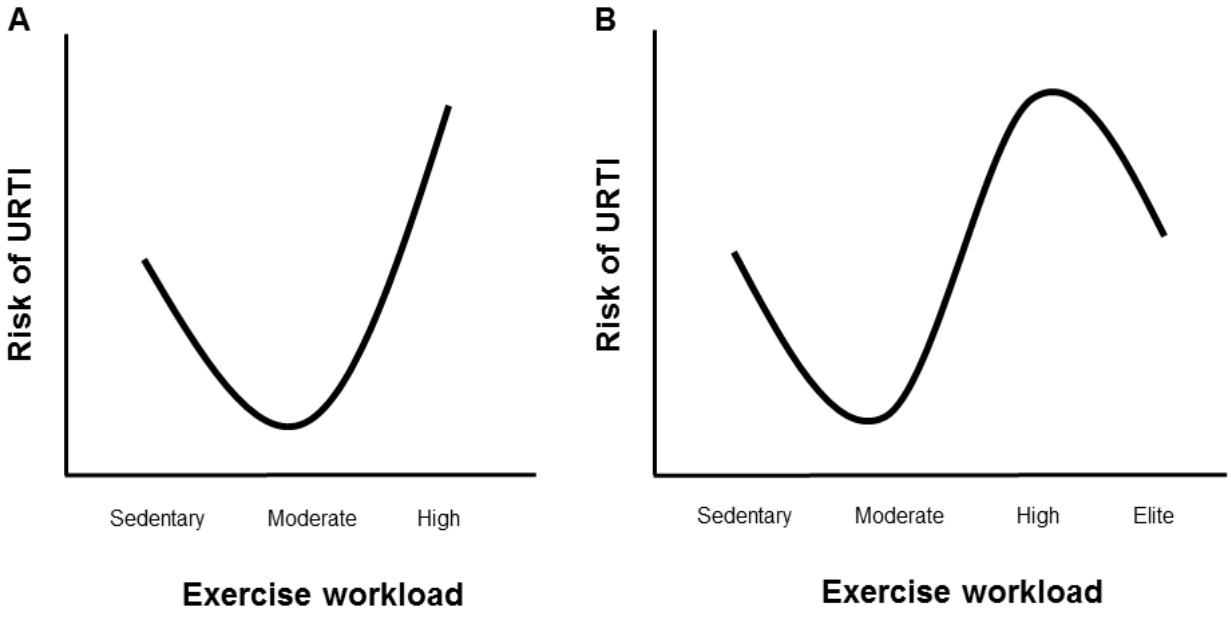

Figure 1.1. The proposed J-shaped (A) (Nieman, 1994) and S-shaped (B) (Malm, 2006) relationship between exercise and risk of URTI.

Nieman (2001) suggested most athletes may not report URI or suffer from an increased risk if they avoid periods of overreaching/overtraining (Nieman et al., 2000). It seems that an increase in URI may only be attributed to participation in acute prolonged exertion (e.g. marathon, ultra marathon) or more importantly when athletes are exposed to a greater strain of training through exceeding individual training thresholds coupled with inadequate recovery or other life stressors (e.g. sleep disturbance) (Foster et al., 1998; Hausswirth et al., 2013; Pyne and Gleeson, 1998).

1.2 Aetiology of upper respiratory illness

In line with the general population, when pathogen identification has been attempted, bacteria are rare causes of URTI in athletes, with viruses being responsible in most cases (in particular rhinovirus, adenovirus and parainfluenza) (Mäkelä et al., 1998; Roberts, 1986). URTI of viral origin last approximately 3-14 days but clearance of the particular virus from the system may take longer (Heikkinen and Järvinen, 2003; Winther et al., 1986). The route of entry into the body by most viruses is the respiratory tract where symptoms reflect the perturbations in function of infected cells and the attempts of the immune system to contain the infection (Roberts, 1986). As the nature of symptoms of some (i.e. infectious) URI may be similar to non-infectious inflammatory factors and/or presentation of allergic conditions, identification of antibody titres and isolation of specific pathogens from body fluids of athletes have been recommended to clarify causes of symptoms and provide the most appropriate treatment or management strategies (Cox et al., 2008; Gleeson, 2006b). As mentioned previously, it was generally believed that upper respiratory symptoms in athletes were due to an infective cause, however, it is only more recently that other

causes (‘non-infectious hypothesis’) have been proposed during training and competition (Bermon, 2007; Schwellnus et al., 2010).

Robson-Ansley et al. (2012) recently reported that a higher incidence of URI in runners following a marathon (47%) compared to non-runners (19%) was significantly associated with positive responses in the Allergy Questionnaire for Athletes. As the prevalence of inhalant allergy may be as common as 16-32% of highly trained athletes, it may partly provide an explanation to incidence of URI (Langdeau et al., 200; Lumme et al., 2003; Schwellnus et al., 2010). Environmental influences have been considered relevant to certain groups of competitive athletes, in particular swimmers who are exposed to chlorine derivatives from swimming pool disinfectants while inhaling large amounts of air above the water surface (Bougault et al., 2009; Piacentini et al., 2007). In addition to inhaled irritants, the combination of high ventilation rate in heavy training and surrounding cold, dry air is another possible source of non-infectious, non-allergenic inflammatory stimuli to URI in some athletes (e.g. runners, cyclists) (Bermon, 2007; Cox et al., 2008).

There is also potential for direct damage in epithelial tissue of the upper respiratory tract and/or fibres of the contracting skeletal muscle following strenuous exercise to contribute to the non-infectious, local and systemic inflammatory origin of URI (Peters, 2004). Schwellnus et al. (1997) reported that the use of an anti-inflammatory agent reduced URI in participants following an ultramarathon event, but as this nasal, buccopharyngeal spray also contained antimicrobial properties it does not provide any conclusive evidence to support a purely non-infectious inflammatory cause for URI. In contrast, administration of an anti-inflammatory throat spray in the period leading up to and following a half-marathon event had no influence on the

incidence of URI in runners but did reduce the severity of recorded symptoms (Cox et al., 2010). Although conflicting evidence here may purely reflect site-specific differences between agents, together these studies do provide evidence that non-infectious inflammation may not be the underlying cause of all URI but may interact with infectious causes to potentiate the symptoms that occur as a result of responses to pathogen challenge.

In their surveillance study, Spence et al. (2007) demonstrated that the distribution of URI closely followed the J-shaped curve in terms of training status (i.e. both the control and elite athlete group suffered from greater days of illness than their recreational counterparts), but only 30% of reported illnesses were confirmed by identification of common respiratory pathogens. It is worthy to note that only specific pathogens were tested in Spence et al. (2007), thus URI were possibly caused by known pathogens not tested for, unknown pathogens and/or new strains of viruses which were yet to be identified (Bermon, 2007). Despite the low number of identified pathogens highlighted with laboratory evaluation of URI in some studies (i.e. Cox et al., 2008; Spence et al., 2007), it must be emphasised that this does not rule out infectious causes for these cases as such diagnostics procedures do have inherent limitations in identifying causative agents from an evolving diverse pool of pathogens (e.g. ~ 200 common cold viruses) (Eccles, 2005; Heikkinen and Järvinen, 2003).

Not all cases of URI can present as a typical response to a primary viral infection of initial upper respiratory symptoms followed by local and systemic inflammation (e.g. fever, aches) (Gleeson et al., 2002). Occasionally symptoms may be minor and/or short-lasting (1-3 days) resulting in training being unaffected or be a reflection of persistent fatigue and recurrent infections as a result of chronic heavy exertion (Reid

et al., 2004). In these cases it has been suggested that, in addition to exercise-induced inflammation, URI following exercise may be related to reactivation of latent viruses within the upper airways rather than the incidence of primary infections in the recovery period (Gleeson et al., 2002). Ekblom et al. (2006) found in a group of recreational runners that pre-race URI was significantly associated with URI incidence following the marathon. This was supported by a recent longitudinal observational field study, where it was suggested that reactivation of pre-race viruses and exercise-induced inflammatory responses were the primary causes of elevated URI incidence prior to and following an 86.5-km Marathon (Peters et al., 2010). The 57% of runners, who recorded incidence of URI during the 7 to 14 days following the race, also recorded symptoms in the time leading up to the race. These findings may support the aforementioned animal studies whereby participation in prolonged exercise may worsen symptom severity induced by existing infection (Malm, 2006).

The viruses reported to be reactivated following strenuous exercise or intense periods of training are the human herpes virus group, primarily cytomegalovirus (CMV) and Epstein-Barr Virus (EBV) (Gleeson et al., 2002; He et al., 2013b). These herpes viruses typically infect humans early on in life, with ~ 50-60% and ~ 80-90% of the world's adult population infected (i.e. seropositive) by CMV and EBV respectively (Bate et al., 2010; Gleeson et al., 2002; Staras et al., 2006). After initial primary infection, these viruses lay dormant within the cells of the immune system and are asymptomatic in immunocompetent individuals (Crawford, 2001; Kano and Shiohara, 2000). However, when significant physical and/or psychological stress is placed on the immune system such latent viruses may become re-activated (Glaser et al., 1999; Mehta et al., 2000b; Stowe et al., 2001; Tingate et al., 1997).

In an exercising population, EBV has received the most attention due to its ability to replicate continuously or intermittently from the orpharynx (Faulkner et al., 2000; Nadal et al., 2002). Gleeson et al. (2002) found a significant correlation between previous EBV infection and URI in elite swimmers while all seronegative swimmers remained unaffected (no reported URI) during a 30 day period of intensive training. Out of the 11 seropositive swimmers, 7 had EBV DNA detected in saliva during the study with 86% of these also reporting URI which appeared 4-18 days following first detection of EBV DNA.

In contrast, He et al. (2013b) in an investigation of 236 endurance athletes found that those seropositive for CMV alone or both CMV and EBV groups had a lower number of days and episodes of URI compared to their seronegative counterparts. Although no differences in URI were observed between EBV seropositive and seronegative athletes, the above findings were contrary to their hypothesis and previous suggestions that prior infection with herpesviruses may predispose athletes to more frequent URI (He et al., 2013b; Hoffmann et al., 2010). There is a need for further research to at least ascertain the relationship between prior infection with CMV/EBV and risk of URI in athletes. Furthermore, systematic DNA analysis of EBV or other members of the human herpes family should be advocated when determining causes of URI in athletes as possible unidentified cases (as seen in Spence et al., 2007) may be related to latent viral shedding (Gleeson et al., 2002).

As mentioned previously, it has been suggested that viral reactivations may be most responsible for shorter episodes of URI than primary infections (Walsh et al., 2011b). Athletes have been found to have greater levels of detectable EBV DNA compared to controls (Hoffmann et al., 2010), where reactivation-induced cell damage and

inflammation have the potential to trigger symptoms in the upper respiratory tract (Cox et al., 2004). In a clinical investigation of elite athletes suffering recurrent episodes of URI, EBV viral shedding was detected in 22% of the cohort (Reid et al., 2004). Yamauchi et al. (2011) also found in an intensive training period with rugby players that salivary expression of EBV DNA was 1.5 times greater in participants with URI compared to those without URI.

Cox et al. (2004) challenged the role of EBV by demonstrating that an anti-herpes virus medication reduced the amount detected in saliva but was not effective in influencing URI over a 4 month training period in distance runners. This leads to an interesting discussion of EBV not being a cause of URI per se, but rather an *in vivo* indication of immunodepression/compromised immunity and therefore, increased risk of developing URI due to other infective causes. Indeed, for the reactivation of EBV to occur, there must be a disturbance within certain parameters of the immune system which usually keep the virus tightly-regulated and dormant. This reflection of immune perturbations within the host (Mehta et al., 2000a) occur in line with the most researched hypothesis (‘open window’) behind an increased risk of primary infections in athletes who participate in prolonged exercise.

The ‘open window’ hypothesis suggests that prolonged/heavy exercise causes depression of the immune system which leaves the body less resistant to viruses and bacteria and thus increases the risk of subclinical and clinical infection for between 3 and 72 h (Nieman, 2001). Nieman and Pedersen (1999) introduced this addition to the J-shaped model (Nieman, 1994) to reflect that the increased risk of URI with heavy exertion was due to a decrease in immunosurveillance and vice versa. The immunological response to acute exercise is deemed a subset of stress

immunology (Hoffman-Goetz and Pedersen, 1994), where responses have been likened to those caused by infection, sepsis, burns or trauma (Pedersen and Hoffman-Goetz, 2000). Although exercise does share some similarities to the hormonal and immunological responses of these clinical physical stressors, there are also important distinct differences in the magnitude of and temporal pattern of responses (Shepard, 2001, 2002). Strenuous exercise induces an ordered sequence of modest changes in pro-inflammatory signalling followed predominantly by anti-inflammatory responses which down-regulates immune function whereas some of the clinical stressors listed above (e.g. sepsis) trigger excessive and overwhelming elevations in systemic pro-inflammatory responses (de Jong et al., 2010; Netea et al., 2003; Shepard, 2002). Exercise immunologists have controlled and adapted the duration and intensity of the stress model of exercise to gain a deeper understanding into how alterations in immune function following acute exercise and training may lead to changes in susceptibility to dormant and primary pathogens.

1.3 Immune system and exercise

The immune system has evolved to protect the human body from pathogens (viruses, bacteria and parasites). It encompasses the ability to maintain homeostasis even when exposed to a wide range of foreign and self molecules (i.e. antigens). The components of the non-specific innate system and the specific acquired system overlap to ensure that a state of immunity against infection is established.

1.3.1 Leukocyte count changes and acute exercise

The number of circulating immune cells (leukocytes) is profoundly influenced by acute exercise with reports from over a century ago highlighting the exercise-induced mobilisation following the Boston Marathon (Larrabbe, 1902). Circulating leukocytes consist of the granulocytes (neutrophils, eosinophils and basophils; 60-70% of total), monocytes (5-15%), dendritic cells (less than 1%) and the lymphocytes (15-25%) which can be divided into innate (Natural killer [NK] cells) and acquired (T helper, T cytotoxic and B cells) cells. It is now clear that leukocytosis which is an increase in the total number of circulating leukocytes (mainly neutrophils and lymphocytes) occurs (up to 400%) during and immediately post-exercise (Simpson, 2013). The observed changes are dependent on the exercise intensity and duration (Gleeson, 2007) with prolonged endurance exercise (> 1.5 h) causing a greater leukocytosis (3 to 4 fourfold increase compared to doubling of neutrophil counts) than brief (20-40 min) high-intensity exercise (Robson et al., 1999b).

At rest, it is estimated that an equal amount of leukocytes within the blood are circulating or within marginated pools, adhered to blood vessel walls of the circulatory system (Athens et al., 1961; Berkow and Dodson, 1987). Foster et al. (1986) suggested the increased cardiac output during exercise and the subsequent

shear stress (increased blood flow) within capillaries induces leukocytes to enter circulation in a process known as demargination. However, it is anticipated that marginal pools within the liver, lung, spleen and other vital organs (e.g. bone marrow, intestines) also contribute to the large leukocytosis following exercise as pools in the lung alone possess lymphocytes which are present in 10 times larger amounts than the circulatory pool (Hogg and Doerschuck, 1995; Simpson, 2013).

Activation of the sympathetic nervous system (elevated concentrations of plasma catecholamines) and the hypothalamic-pituitary-adrenal (HPA) axis (cortisol release) during prolonged exercise also play an integral role in exercise-induced leukocytosis (Anane et al., 2009; Atanackovic et al., 2006; Nieman, 2001). The immediate leukocytosis, particularly neutrophilia (increased neutrophil count) upon onset of exercise is suggested to be due to the haemodynamic and catecholamine induced demargination of vascular and pulmonary pools but this is later followed by a cortisol-induced release of neutrophils from the bone marrow otherwise known as delayed leukocytosis (Allsop et al., 1992). This effect is seen simultaneously with the immediate leukocytosis during prolonged exercise of > 1 h as cortisol level is sufficiently elevated and its action is prolonged, explaining the previously mentioned greater leukocytosis than following shorter, higher intensity exercise (Robson et al., 1999b). Neutrophils released from the bone marrow following stimulation by cortisol are suggested to include a greater proportion of immature cells (e.g. band cells) compared to neutrophils that demarginate from the endothelial walls upon onset of exercise which have similar maturity levels to those already in circulation (Hetherington and Quie, 1985; McCarthy et al., 1991). Although effects on dendritic cells remain unclear, the bone marrow is also a source of maturation for both monocytes and B cells where monocytes also increase proportionately with exercise

duration and B cells are associated with limited redistribution (Lancaster et al., 2005a; Nieman et al., 1998; Okutsu et al., 2008; Pedersen et al., 1990; Shek et al., 1995).

NK and T cells (mostly cytotoxic) also increase proportionately with exercise intensity and duration unlike neutrophils where increases are predominantly influenced by duration (Campbell et al., 2009; Gabriel et al., 1991; McCarthy and Dale, 1988; Shek et al., 1995). Even though lymphocytes are present at numerous sites within the body, evidence suggests that mobilisation of these cells during exercise mainly occurs from secondary lymphoid organs e.g. spleen, intestinal Peyer's patches (PP) rather than primary lymphoid organs such as the thymus or bone marrow (Campbell et al., 2009; Simpson et al., 2007; 2008). The spleen is considered to be an abundant source of the lymphocytes deployed during exercise (Baum et al., 1996; Nielsen et al., 1997), with shear stress and catecholamines being important release mechanisms (Benschop et al., 1993; Dmitrov et al., 2010; Kappel et al., 1991; Shepard, 2003; Timmons and Cieslak, 2008). There is a clear consensus developing that exercise triggers a redistribution of T cells with a longer history of antigen exposure rather than naive T cells (Campbell et al., 2009; Simpson, 2011).

Following the lymphocytosis during and upon completion of prolonged exercise, the high concentrations of adrenaline and cortisol often cause lymphocytopenia (lymphocyte count below resting levels) during the recovery (Nieman, 2001; Nieman et al., 1994). This biphasic response (lymphocytosis during exercise and lymphocytopenia during recovery) has been found with various protocols requiring heavy/prolonged exertion or exhaustive exercise (e.g. Fry et al., 1992; Shek et al., 1995), with lymphocyte count up to 60% below resting levels reported post-exercise

(Simpson, 2011). Lymphocytopenia is due to the selective extravasation of lymphocyte subsets, NK and T cytotoxic cells, from blood to the surrounding tissues (Gabriel et al., 1991; Kruger et al., 2008; Simpson et al., 2006). The return of total leukocyte count to resting levels generally begins immediately post-exercise with diminishing activation of the sympathetic nervous system and HPA axis but in the case of very intense exercise, leukocyte (primarily neutrophils) count may continue to increase as described above (Allsop et al., 1992; McCarthy and Dale, 1988). Given these leukocyte perturbations, an increase in neutrophil:lymphocyte ratio is suggested to be an indicator of the overall magnitude of the stress response induced by exercise (Nieman, 1998).

1.3.2 Innate immune cell function and acute exercise

The immune system consists of many physical barriers (e.g. skin, mucus, cilia) which act to prevent entry of pathogens into the human body. If such attempts fail, infectious agents will be detected by receptors present on the cellular components of the innate immune system such as the granulocytes (mostly neutrophils but include basophils and eosinophils), NK lymphocytes, monocytes (which mature into macrophages within tissue) and dendritic cells.

The recognition of foreign material is one way in which the innate and acquired immune systems differ from one another. Unlike acquired immunity, the innate system does not exhibit memory of previous encounters with antigens (i.e. foreign molecule), therefore a similar response is believed to take place during any future exposure. Present on the cell surface of innate cells are pattern recognition receptors (PRR) that distinguish self from non-self material by recognising molecules on microbes which have been conserved through evolution due to their essential

function i.e. bacterial DNA, lipopolysaccharide (LPS) and other bacterial cell wall components (Beutler and Rietschel, 2003; Kimbrell and Beutler, 2001). The majority of these structures comprise of the Toll-like receptors (TLR) which are crucial components of the antigen presentation cells (APC) (discussed later in this section) of innate immunity (monocytes/macrophages).

Neutrophils also known as polymorphonuclear (PMN) cells (due to their multi-lobed nucleus) are the most abundant circulating leukocyte population of the immune system. These leukocytes are considered specialised short-lived cells with deficits in numbers and/or function of this subset being associated with increased risk of potentially fatal bacterial infections (Boxer, 2003; Smith et al., 1996a; Viscoli et al., 2005). Once released from the bone marrow, neutrophils migrate out of the circulation within 4-10 h to marginated and tissue pools where they reside for a further 1-2 days (Smith, 1994; Summers et al., 2010).

Neutrophils respond to chemotactic stimuli (e.g. formylated peptides such as formyl methionyl leucyl phenylalanine, fMLP) from fragments of invading microorganisms and tissue damage at sites of infection and inflammation respectively where they engage in numerous intrinsic conformation adaptations during and following their migration (chemotaxis) (Smith and Pyne, 1997). Similar to other phagocytes (e.g. APC, discussed later in this section), the detection of pathogens or tissue damage through PRR (e.g. FPR1, a fMLP receptor) on the neutrophil surface leads to internalisation (phagocytosis) and holding of these fragments within the cytoplasm (via a membrane bound vesicle known as phagosome) thereby activating a series of effector functions in the cell (Borregaard, 2010; Nathan, 2006). The mechanistic detail of engulfment by neutrophils may depend on the involvement of the specific

ligand (i.e. antigen) attached to the receptors or whether fragments have been opsonised by soluble immune factors but the process will ultimately involve internalisation into a phagosome (Amulic et al., 2012). Soluble components which act as opsonins (promote attachment of antigen to phagocyte) include acute phase proteins, antibodies (see section 1.3.3) and complement proteins (e.g. C3) (Brekke et al., 2007; Gordon, 2002; Thiel et al., 1992). It is worthy to note that elevations in circulating concentrations of acute phase proteins (c-reactive protein, CRP) in response (i.e. acute phase response) to perturbations in homoeostasis (e.g. trauma, tissue damage) are considered one example of why the strenuous nature of prolonged exercise is likened to medical conditions (e.g. sepsis, burns) (Fallon, 2001; Kushner and Rzewnicki, 1994; Pedersen and Hoffman-Goetz, 2000).

Neutrophils are also known as granulocytes due to the characteristic release (degranulation) of primary azurophil (defensins, elastase, myeloperoxidase [MPO]) and secondary specific (lactoferrin, lysozyme) granule contents (degranulation) into the cytoplasm to fuse with the phagosome or the plasma membrane to create an antimicrobial milieu inside and outside of the cell. These granules do show varying readiness to mobilise in response to inflammatory signalling, with the azurophilic subset being the most difficult to mobilise (Amulic et al., 2012). The degradation of particles within the phagosome or extracellular space of infected or damaged tissue is aided further by the process of neutrophil oxidative burst involving the formation of reactive oxygen species (ROS) through the NADPH oxidase system (Babior, 1999; Weiss, 1989). Depending on the stimulus (see section 2.4.3.1 for relevant stimuli of this thesis), the NADPH oxidase system may assemble on the membrane of the phagosome and/or the cell where the main ROS produced are the superoxide anions which react to form other intermediates including hydrogen peroxide, hypochlorous

acid, hydroxyl radical and singlet oxygen (Peake, 2002). It has been suggested that components of azurophilic (e.g. MPO) and specific (e.g. flavocytochrome b558) granules may regulate the activity of the NADPH oxidase (Tal et al., 1998; Amulic et al., 2012), highlighting that intracellular signalling cascades triggered by attachments of ligands to surface receptors, may lead to coordinated degranulation and oxidative burst responses to invading pathogens (Daniels et al., 1994; Pyne, 1994).

Recently, it has been demonstrated that neutrophils also possess antimicrobial capacity independent of phagocytosis known as neutrophil extracellular traps (NETs) (Mantovani et al., 2011). Although mechanisms of NETs are not completely understood, it has been established that classical effector functions of neutrophils (degranulation and oxidative burst) play important roles in mediating the response (Amulic et al., 2012; Fuchs et al., 2007; Metzler et al., 2011; Patel et al., 2010). NETs are suggested to form upon an active form of cell death which results in the release of a network of nuclear filaments (DNA and histone) into the extracellular space from the degrading neutrophil (Brinkmann et al., 2004; Fuchs et al., 2007).

On the basis of the exercise-induced changes in neutrophil counts it is not surprising that exercise also affects functional responses, but due to the variation in study design the early evidence was conflicting (Peake, 2002). Nevertheless, it can be argued that when responses are controlled for on a per cell basis the effects of exercise duration (i.e. prolonged exercise) are clearer. Neutrophils may present on a continuum of state of activation from dormant to primed through to being fully activated (Smith, 1994). During exercise, there is a release of agents into the circulation which may prime (e.g. induce assembly of NADPH on membrane) or desensitise (internalisation of receptors) the capacity of neutrophils for enhanced

responsiveness to later stimulation or inhibit such functional responses respectively (Amulic et al., 2012; Peake, 2002; Pyne, 1994).

The number of neutrophils engaging in phagocytic activity is increased following prolonged exercise but the phagocytic capacity of each neutrophil is decreased within the circulation and the nasal cavity (Blannin et al., 1996a; Chinda et al., 2003; Gabriel et al., 1994; Muns, 1994; Nieman et al., 1998). Albers et al. (2005) suggested that neutrophil phagocytosis may have low suitability as a marker of the immunomodulatory effects of exercise and assessing the degranulation and/or oxidative burst may be more sensitive to reflect a susceptibility to infection.

Simultaneous with neutrophilia following prolonged exercise there is an increase in unstimulated degranulation and oxidative burst responses (e.g. measured by total plasma elastase, spontaneous ROS production) (Bishop et al., 2003; Blannin et al., 1996b; Suzuki et al., 1999). These findings provide support that prolonged exercise induces neutrophil activation (possibly due to muscle damage) that may result in the cells entering a ‘refractory period’ in the recovery from prolonged exercise whereby there is a transient window of an inability to respond to subsequent stimulation (e.g. *in vitro*) (Peake, 2002).

Numerous studies have found significant decreases in neutrophil degranulation and/or oxidative burst responses to *in vitro* stimulation by bacterial peptides (fMLP and LPS) and/or synthetic stimuli (Phorbol-12-Myristate-13-Acetate, PMA) during the recovery from prolonged (> 1.5 h) exercise (Chinda et al., 2003; Davison and Diment, 2010; Davison and Gleeson, 2005, 2006, 2007; Davison et al., 2007; Laing et al., 2008; Suzuki et al., 2003). This decline in function has not been coupled to changes in cell surface receptors, suggesting that the modulatory effects of exercise

on *in vitro* stimulants occur downstream in intracellular signal transduction pathways of neutrophils such as phosphorylation cascades or secondary messengers (e.g. cyclic adenosine monophosphate, calcium) (Mooren et al., 2001; Peake, 2002). The magnitude of the effects of prolonged exercise on neutrophil effector functions will depend on the balance of immunosuppressive (e.g. 'refractory period') and immunostimulating factors (e.g. priming agents) which largely depend on the extent of neutrophilia (Peake, 2002). As previously mentioned, prolonged exercise (i.e. elevations in stress hormones) induces a mobilisation of immature neutrophils into the circulation which have been shown to exhibit reduced NADPH oxidase activity and granular content (Berkow and Dodson, 1986: Hetherington and Quie, 1985). The immunodepressive effects of cortisol and the subsequent release of subpopulations from the bone marrow was supported by Robson et al. (1999b) who found that the degree of exercise-induced decrease in stimulated degranulation per neutrophil and neutrophilia was greater following prolonged exercise (~ 3 h) compared to short, intense exercise (~ 40 min).

Although considered to be a major factor, activation of the HPA axis alone cannot wholly account for changes in the neutrophil function (Laing et al., 2008), as some of the noted inhibitory effects of cortisol on receptor mediated responses (e.g.fMLP) would not explain decreased responses to stimulants that activate neutrophils independent of surface receptors (e.g. PMA) (O'Flaherty et al., 1991; Peake, 2002; Tomchek et al., 1991). Thus, other mechanisms have also been suggested to be involved in neutrophil dysfunction, where elevations in catecholamines, cyclic adenosine monophosphate, complement proteins (C5a), direct cellular oxidative damage and growth hormone are responses which may occur with the inflammatory response to prolonged exercise and have been shown to interfere with calcium

signalling or other intermediates of intracellular pathways (Hack et al., 1994; Henson et al., 1978; Laing et al., 2008; Robson et al., 2003; Suzuki et al., 1999; Thibault et al., 2000; Tintinger et al., 2001).

The susceptibility of phagocytes to modulation by strenuous exertion are also demonstrated by the decreased expression of TLR on the cell surface of monocytes immediately and up to 2 h following prolonged exercise (Lancaster et al., 2005b; Oliveria and Gleeson, 2010) which are not explained by exercise–induced changes in monocyte numbers alone (Simpson et al., 2009). The phagocytic function of monocytes has been shown to increase following prolonged exercise which may be related to the increased pro-inflammatory phenotype observed with exercise in this leukocyte (Hong and Mills, 2008; Steppich et al., 2000). Once monocytes reach the tissues they will form mature macrophages, therefore the biological significance of changes in monocytes is currently unclear as it may not reflect exercise-induced changes in immunosurveillance within tissue (i.e. at sites of inflammation or infection) (Simpson et al., 2009; Walsh et al., 2011b). These macrophages along with other phagocytes (dendritic cells) are present in majority of body tissues where unlike the direct killing capacity of neutrophils, macrophages and dendritic cells mostly act as professional APC (Beutler, 2004; Iwazaki and Medzhitov, 2004) . Investigations within exercise stress models thus far have been limited to animal studies which are difficult to generalise to the human response but do suggest dysfunction in the capacity of these cells to present antigens following prolonged exercise (Ceddia and Woods, 1999; Ceddia et al., 2000; Chiang et al., 2007; Davis et al., 1997; Liao et al., 2006; Murphy et al., 2004; Woods et al., 1997, 2000).

NK cells are large granular cells that can sense structures of high-molecular weight glycoproteins expressed on virus infected cells via PRR on their cell surface. They form up to 15% of the lymphocytes within the body and are vital in defence against viral infection. However, compared to other peripheral lymphocytes (B and T cells, see section 1.3.3), prior sensitisation is not required (Cerwenka and Lanier, 2001). Thus upon activation NK cells trigger apoptosis or lysis of a virus-infected cell by releasing granule contents such as the pore forming proteins perforin and cytolysin. The initial investigations into NK cell activity (NKCA) showed that exercise-induced responses were largely mediated by the duration and intensity of the bout (Gannon et al., 1995). The NKCA was demonstrated to mirror the increases in NK cells following moderate or exhaustive exercise (Gannon et al., 1995; Woods et al., 1998). However, when the exercise bout is intense and prolonged, NCKA on a per cell basis has been shown to be reduced for several hours (Kappel al., 1991; McFarlin et al., 2004; Nieman et al., 1993).

1.3.3 Acquired immune cell function and acute exercise

Binding of microorganisms to PRR (e.g. TLR) of innate immune cells not only triggers activation of innate parameters but also induces the generation of protein messengers (i.e. cytokines) and other signalling components to stimulate acquired immunity (Takeda and Akira, 2005). However, compared to innate immunity, the acquired immune response to a pathogen is delayed due to a lag period for recognition by the vast range of antigen receptors within B and T lymphocyte populations, clonal selection/expansion of the pertinent lymphocytes and clonal elimination for tolerance of self to occur (Kimbrell and Beutler, 2001; Medzhitov, 2001).

T and B cells form 60-80% and 5-15% of the circulating pool of lymphocytes respectively, where the T cell population mature in the thymus gland and the B cells in the bone marrow. T lymphocytes are subdivided further into cytotoxic (T^c, CD8+), helper (T^h,CD4+) and regulatory (T^{reg}) where the T^{reg} are formed from a naive CD4+ cell and modulate immune responses compared to the primary involvement in removal of pathogens by T^c and T^h.

The induction of a primary immune response to pathogens involves presentation of antigens to the cell surface receptor of T lymphocytes (T cell receptor) by dendritic cells (Lanzavecchia and Sallusto, 2001; Mellman and Steinman, 2001), while all other APC (e.g. monocytes, macrophages) are involved in the initiation of secondary immune responses via memory T cells which have encountered the antigen previously (Gallucci and Matzinger, 2001). T cells are able to recognise antigens via Major Histocompatibilty Complex (MHC) class I and II molecules on the cell surface of an APC (Banchereau and Steinman, 1998). The number of antigens encountered by an individual will determine the proportion of naive (unactivated) or memory (activated) T cells circulating within the body.

Although structurally similar to the T cell receptor, the surface receptor of the B lymphocyte is a membrane-anchored immunoglobulin (Ig) (also termed antibody). Upon interaction of antigen and receptor, the B cell undergoes clonal expansion to form both short-lived plasma effector cells and long-lived memory cells or internalise the bound antigen and act as an APC to T cells (LeBien and Tedder, 2008). The short-lived plasma cells secrete Igs into the blood to aid destruction of the pathogen while the memory cells with their greater affinity to the antigens can remain in the

circulation throughout life to rapidly differentiate into plasma effector cells if the same antigen is encountered again (Walsh et al., 2011b).

The circulating Igs fall into five major classes, IgM, IgG, IgA, IgD and IgE where each of these classes can be divided further into subclasses. This diverse repertoire of Igs possess functional regions capable of binding to a vast range of antigen sites (epitopes) to form immune (antigen-antibody) complexes that neutralise the toxicity of certain antigens or common Ig regions which can activate phagocyte ingestion and soluble innate factors (e.g complement) (Nieman and Nehlsen-Cannarella, 1991). Following a lag period for accumulation of Igs within the blood, the IgM class predominates during any primary response to an antigen whereas IgG predominates under normal resting conditions as well as in the rapid Ig response to secondary antigen exposure (McKune et al., 2005; Stavnezer, 1996).

Therefore, the acquired immune system consists of a cellular (i.e. T cells) and humoral component (i.e. Ig). Determining which component of the acquired immune system predominates, are the subpopulations of T^h cells (T^h1 and T^h2) and the cytokine profile they produce and release (Walsh et al., 2011b). Cytokines act as protein messengers between one immune cell and other, where the cell receiving the signal may proliferate, secrete additional cytokines, migrate to the area of origin of the signal, differentiate into another type of cell or die (undergo apoptosis) (Curfs et al., 1997). The main cytokine group responsible for leukocyte communication is the interleukin (IL) family but other major cytokine groups are the colony stimulating factors (e.g. granulocyte macrophage colony stimulating factor [GM-CSF], granulocyte-colony stimulating factor [G-CSF]), tumour necrosis factors (TNF) and

interferons (IFN) where their main roles are stimulation of cell growth, tumour cytoxicity and inhibition of viral replication respectively (Curfs et al., 1997).

The stimulation of T^h1 cells and production of cytokines such as IL-2, and IFN γ promotes cell-mediated immunity (T^c responses) for defence against intracellular pathogens while activation of T^h2 cells (IL-4, IL-5, IL-6 and IL-13) coordinates humoral immunity (B cells) and release of Igs for defence against extracellular pathogens (Seder, 1994). It has been suggested that intense/prolonged exercise can modulate this balance by decreasing the proportion of T^h1 cells in circulation whereas T^h2 cells remain unaffected (Lancaster et al., 2004, 2005a; Steensberg et al., 2001). This shift in immunity has been proposed to provide an explanation for potential increases in the susceptibility to URI following prolonged exercise given the importance of type 1 responses (T^h1) towards viral infection (Fabbri et al., 2003; Steensberg et al., 2001).

Such effects of exercise are suggested to be mediated via the suppression of type 1 cytokine production by elevations in stress in hormones (adrenaline and cortisol) and the release of cytokines from contracting skeletal muscle (primarily IL-6) that favour production of type 2 (Th2) cytokines (Gleeson, 2007). Supporting evidence for cytokine modulations following intense exercise has been provided by decreases in IL-2 and IFN and no changes in IL-4 shown with *in vitro* mitogen-stimulated isolated cells and whole blood culture (Moyna et al., 1996; Smits et al., 1998; Starkie et al., 2001; Tvede et al., 1993). These changes in cytokine production overlap with influences on other stages of T cell activation where decreases in mitogen-induced T cell proliferation have been observed following intense/prolonged exercise (Bishop et al., 2005; Fry et al., 1992; Henson et al., 1998; Nieman et al., 1994, 1995).

Despite these observations, the evidence suggesting that T cell activation is down-regulated by prolonged exercise is confounded by numerous methodological limitations (Walsh et al., 2011b). T cell proliferation assays generally use a fixed amount of total lymphocytes or whole blood so changes following exercise may only reflect changes in the proportion of lymphocyte subsets due to the greater increase in NK cells post-exercise which do not respond to mitogen (Green and Rowbottom, 2003). Depletion of NK cells from cell culture has been found to remove the significance of changes in mitogen-induced proliferation following exercise (Green et al., 2002). Nieman et al. (1994) found that proliferative responses were substantially different based on adjustments for T cell populations, but there was still a significant fall in proliferation following intense exercise compared to pre-exercise. Nevertheless, the validity and sensitivity of mitogen-induced culture to assess T cell function have been questioned due to their non-specific effects (activating other cell types e.g. B cells) and lack of ability to detect subtle changes in individual T cell populations (Bishop et al., 2005).

Bishop et al. (2009) demonstrated that *ex vivo* migration of CD4+ and CD8+ cells to a human-rhinovirus infected bronchial (lung) epithelial cell line was decreased following a prolonged exercise bout (2 h of running). It is also difficult to extrapolate such observations on isolated cells from a circulating pool that is considerably lower than total lymphocyte mass within the complex *in vivo* environments at the skin, mucosa, and lymph nodes (Gleeson, 2007). Although investigations on whole blood maintains the proximity of leukocytes and the extracellular milieu of leukocytes compared to leukocyte isolation, the use of *in vivo* measures to assess response to antigenic challenge may be more clinically relevant (Albers et al., 2005; Walsh et al., 2011b).

Delayed Type Hypersensitivity (DTH) is a complex local immunological reaction to an intracutaneous antigen (following previous encounter/sensitisation) that is primarily T-cell-mediated but involves interaction of various cell types and chemical mediators of the immune system to induce a cell mediated immune response (Albers et al., 2005; Harper-Smith et al., 2011). Although the antigen presentation to T cells within a DTH reaction is similar to response to intracellular pathogens (Kaufmann, 1995), it can be considered an amplified reflection of the stages that occur with a 'conventional' antigen which allows for the cell-mediated immune response to be non-invasively quantified (Kobayashi et al., 2001; Harper-Smith et al., 2011). The DTH reaction is characterised by an infiltration of predominantly peripheral mononuclear cells (PBMC: T cells (antigen specific) and monocytes/macrophages) which release cytokines and other inflammatory mediators that present as eczematous plaques at the site (i.e. skin) (Bruunsgaard et al., 1997; Kaplan et al., 2012; Malaijan and Belsito, 2013; Schwarz, 2003). Such epidermal indurations (oedema and erythema) that peak in the 24-48 h following antigen re-exposure can then provide the means by which cell mediated immune response can be measured (Albers et al., 2005).

Bruunsgaard et al. (1997) showed that triathletes who completed prolonged exercise prior to intra-dermal injections of 7 previously encountered antigens had significantly reduced skin responses compared to resting controls, indicating immunodepression. This method, however, is limited to the recall (elicitation) of pre-existing immunological memory and does not provide any evidence on the effects of prolonged exercise on the primary immune (induction) response to a novel antigen (Harper-Smith et al., 2011). Contact sensitisation which involves topical skin exposure to novel synthetic chemicals provides an experimental model where both

the elicitation and induction of T-cell-mediated responses can be measured (Albers et al., 2005; Friedmann, 2007; Palmer and Friedmann, 2004).

Harper-Smith et al. (2011) found that participating in prolonged exercise (2 h of running) compared with seated rest prior to cutaneous sensitisation with a novel antigen (Diphenylcyclopropenone, DPCP) reduced the recall (elicitation) of the immune response (skin erythema and oedema) to that same antigen 4 weeks later. When participants completed the same prolonged exercise immediately prior to the elicitation phase (recall at 4 weeks), there was also a reduction in skin responses to DPCP compared to resting controls. However, the magnitude of reduction in response to DPCP at this phase (elicitation, 20%) was considerably lower than the reduction observed when prolonged exercise was completed prior to DPCP sensitisation (induction, 50%). This suggests that the primary immune response to antigens (APC and induction of T cell specific memory) is more susceptible to the perturbations induced by prolonged exercise than responses to recall (previously exposed) antigens.

Simultaneously to the aforementioned assessment of cell-mediated immunity, Bruunsgaard et al. (1997) also vaccinated all groups with previously encountered antigens that would stimulate B cell function. There were no differences between the prolonged exercise and control group in the ability of B cells to generate antibody responses to these recall antigens in the 14 days following vaccination. This may provide supporting evidence to proposals that primary immune responses are more susceptible to the effects of exercise or it may suggest that perturbations are more pronounced in the immediate period following prolonged exercise (0-48 h, i.e. proposed open window). The response of circulating Igs to prolonged exercise has

not been extensively researched with the studies that have investigated the predominant Igs in blood (A, G, M) reporting conflicted findings (decreases, increases and no change) (Bishop, 2013; Nieman and Nehlsen-Cannarella, 1991; Peters et al., 2010; Walsh et al., 2011b). This may not be surprising given the small proportions of B cells (5-15%) in the circulating lymphocyte pool and little redistribution of this subset following exercise. For the investigation of activated B-cells and Ig responses to exercise, studies have focused primarily on other immune compartments which may have greater relevance to URTI (i.e. mucosa).

1.3.4 Mucosal immunity and acute exercise

Both cell-mediated and adaptive parameters contribute to the largest component of the immune system, mucosal immunity (total surface area of 400 m^2) (Brandtzaeg et al., 1999). The importance in host defence against pathogens becomes apparent when recognising that the mucosal surfaces of the upper and lower respiratory tract account for ~ 50-60% of total immune protection by the body and the small intestine along with the colon are responsible for 70% of all Igs produced (Kudsk, 2002). Increases in illness and morbidity have been attributed to impairment in mucosal immunity (Daele and Zicot, 2000), highlighting the importance that immune competence at mucosal surfaces has in the health and well-being of athletes (West et al., 2006). Although not functioning independently of the systemic immune system, mucosal immunity is also considered a distinct entity due to its autonomously regulated, localised defence mechanisms (Toy and Mayer, 1996).

The gut-associated lymphoid tissue (GALT), urogenital tracts, lacrimal glands, lactating mammary glands and respiratory tracts which include the bronchus-associated lymphoid tissue (BALT), salivary glands and nasal-associated lymphoid

tissue are all mucosal surfaces which fall under the network of immune structures known as the common mucosal immune system (CMIS) (Gleeson and Pyne, 2000). The immunological protection provided by this network may be via organised tissue with well-formed follicles (mucosa-associated lymphoid tissue) such as PP of the small intestine or as a diffuse accumulation of leukocytes (lymphocytes, plasma cells and phagocytes) as found in the lung and the lamina propria (connective tissue) of the small intestine (Kyd and Cripps, 1999). These immune structures are pivotal for the mono-layered epithelial layer of mucosal surfaces which is continually exposed to a wide array of antigens or allergens including pathogenic bacteria or viruses, gut microflora and ingested food (Johansen et al., 2000). Indeed, the mucosae are considered to be the first line of defence as they are the sites where most pathogens enter the body (Macpherson et al., 2012).

The CMIS are differentiated into inductive and effector sites, where the induction sites (primarily PP) involve the sensitisation of immune response following antigen presentation while the effector sites consist of interconnected distal sites (respiratory tract, lamina propria) where the array of activated B-cells and plasma cells home and migrate to provide local protection (Kyd and Cripps, 1999; Kudsk, 2002). At least 80% of the body's plasma cells (activated B-cells) reside in the mucosal effector tissues, whereby local production of Igs represent a major immunological barrier at all mucosal surfaces with IgA being the predominant antibody (Bishop and Gleeson, 2009; Brandtzaeg et al., 1999).

Within the bloodstream, IgA under most circumstances is found as a monomeric peptide (Yel, 2010). However, in all mucosal secretions IgA exists as a dimeric protein covalently linked by a J chain containing another peptide termed the

secretory component (Gleeson and Pyne, 2000). The secretory component is the cleaved segment of the polymeric Ig receptor (pIgR) that is produced by the mucosal and glandular epithelial cells and expressed on the basolateral membrane (Bishop and Gleeson, 2009; Teeuw et al., 2004). The proteolytic cleavage of pIgR occurs following its binding and induction of the active transport (exocytosis) of dimeric IgA through epithelial cells on to the mucosal surface (Strugnell and Wijburg, 2010). The remaining secretory component wrapped around the J chain-linked dimeric IgA forms secretory IgA (SIgA) which is considered to be resistant to proteases secreted at mucosal sites (e.g. intestinal mucosa) (Johansen et al., 2001; Lindh, 1975; Strugnell and Wijburg, 2010; Underdown, 1974). It is this local production of SIgA that forms the major effector function of mucosal immunity (Bishop and Gleeson, 2009). Only in the neonate or situations of IgA deficiency does IgM represent a significant defence at mucosal surfaces with IgG also being found in low quantities at mucosae (Brandtzaeg et al., 1999; Gleeson and Pyne, 2000).

IgA can be further divided into subclasses, where IgA2 is the most abundant in the distal gastrointestinal tract (60%), whereas IgA1 predominates in the salivary glands (60% - 80%) and nasal lymphoid tissue (NALT) (> 90%) (Gleeson, 2000). Protection of mucosal surfaces via SIgA occurs through multiple mechanisms. One such mechanism known as immune exclusion involves the binding of antigens to regulate the commensal microorganisms (microbiota) and prevent the attachment and invasion of mucosal surfaces by pathogens (Strugnell and Wijburg, 2010; Sutherland and Fagarsan, 2012). Other mechanisms include the binding of antigens which have already crossed the mucosal barrier and actively transporting them back across the epithelial layer into the lumen or intracellular neutralisation of viruses when bound to pIgR within the mucosal epithelia (Lamm, 1998).

The presence of SIgA (formed by B cells adjacent to salivary ducts and glands) in saliva has tended to be the mucosal immune marker of choice due to the ease of collection (Bishop and Gleeson, 2009; Gleeson, 2000; Korsrud and Brandtzaeg, 1980; Sari-Sarraf et al., 2006). Most of the salivary fluid itself is formed by 3 pairs of major salivary glands (parotid, submandibular, sublingual) but production is supplemented by a vast amount of small submucosal glands that lie on and around the tissue (e.g. palate, tongue) within the oral cavity (Proctor and Carpenter, 2007). Although saliva drains from the acini (cluster of cells) of each of these glands into the mouth via striated and excretory ducts, the nature of the secretion differs whereby a serous (watery) fluid is produced by the parotid, mucous fluid by the submandibular and sero-mucous mixture by the sublingual (Aps and Martens, 2005). In unstimulated saliva secretion, the proportion of the fluid provided by parotid, submandibular, sublingual and the remaining submucosal glands are on average suggested to be 25%, 60%, 8% and 8% respectively (Dawes, 2008).

In addition to the aforementioned transcytosis of SIgA into the mucosal secretion, saliva benefits from the import of several antimicrobial peptides (AMPs) that contribute to the first line of defence by providing innate defences compared to the specific nature of IgA (Bals, 2000). AMPs are categorised as being small cationic peptides (< 100 amino acids) that represent inducible, constituent factors of mucosal secretions (West et al., 2006). Although numerous AMPs require some form of enzymatic modification prior to their functional configuration, they act in synergy with other components of the innate immune system to prevent and aid clearance of infections (Bowdish et al., 2005; De Smet and Contreras 2005; Ibrahim et al., 2005; Radek and Gallo, 2007; Wakabayashi et al., 2003). Several AMPs can modulate other immune processes such as leukocyte cytokine secretion, chemotaxis and

remodelling of injured epithelia (Bowdish et al., 2005; Ganz, 2003; Tjabringa et al., 2005).

The most abundant AMPs in the secretions of the upper respiratory tract are lysozyme and lactoferrin (Singh et al., 2000). Lysozyme is a small protein (145 kDa) released into saliva by neutrophils, macrophages and the submucosal glands that possesses antibactericidal capacity through hydrolysing the polysaccharide of bacterial cell walls (Bosch et al., 2002; Fabian et al., 2012; Jolles and Jolles, 1984; Travis et al., 2001; West et al., 2006). The antimicrobial properties of the smaller molecule lactoferrin (80 kDa) from neutrophils and submucosal glands are due to its ability to bind free iron, depriving bacteria of this nutrient that is essential for growth and multiplication (Bowdish et al., 2005; Legrand et al., 2004; Ward et al., 2005). Although not an inhibitor of the main causative agent of URTI (rhinovirus), lactoferrin is considered to be effective against other common respiratory viruses (adenovirus, respiratory syncytial virus (West et al., 2006).

There is a wide variety of other AMPs, most of which have been grouped into 3 main families; cathelicidins (e.g. LL37), defensins (α and β sub-families) and histatins (Bals, 2000). These are primarily released into the oral cavity by the epithelial cells, salivary glands, and/or neutrophils (De Smet and Contreras, 2005; Fabian et al., 2012). The AMPs work synergistically in low concentrations to destabilise cell walls of microorganisms and provide a broad spectrum of activity against gram-positive and gram-negative bacteria (Bals, 2000). Other salivary proteins that contribute an important line of defence via the inhibition of adherence and growth of specific bacteria (e.g. *Streptococcus*) at the oral mucosa include α-amylase (Scannapieco et al., 1994).

In addition to influx of invading microorganisms from the external environment, the human mouth has a constant microbial presence that needs to be regulated (Bender et al., 2006). Surfaces of the oral cavity are bathed with saliva, whereby the fluid is recognised to provide a 'fingerprint' of the vast range of the resident microorganisms (Boutaga et al., 2007; Dewhirst et al., 2010; Fabian et al., 2008; Li et al., 2005). Reduced salivary flow rate can directly impact on the oral microbiome by inducing a shift towards colonisation via pathogenic microorganisms (Meurman, 2012). Indeed, the entire surface of the respiratory tract is a source of commensal microorganisms which similar to exogenous antigens possess the ability to become pathogenic in the host (Bosch et al., 2013; Watson et al., 2006). AMPs not only play a crucial role in the protection against foreign pathogens but also act as a synergic arsenal of molecules that regulate the response to commensal bacteria (Boman, 1995). Therefore, the importance of AMPs is twofold; preventing disruption of the epithelial layer by acting as a critical first line of defence (biofilm) against pathogens and maintaining homeostasis of the commensal community that act to outcompete invading microorganisms (Blaser and Falkow, 2009; He et al., 2013a; Murphy et al., 2009).

The overlapping nature of these defences is highlighted when reduction in expressions of AMPs in *in vitro* models lead to changes in bacterial colonisation (Bals, 1999; Liu and Modlin, 2008). Subsequently, perturbations in the balance of the microbiota on the mucosal surface can lead to overgrowth and further amplification of microbes which may have direct effects locally (e.g. oral infection, URTI) or have indirect effects through predisposing respiratory illness with the many established interactions between microorganisms (bacteria-bacteria, virus-bacteria) (Blaser and Falkow, 2009; Bosch et al., 2013; Murphy et al., 2009; Meurman, 2012; Slots and

Genco, 1984). Such debilitating effects are likely to occur if the imbalance in microbiota occurred due to a lack of immune competence (e.g. decreased AMPs) (Bosch et al., 2013).

The resistance of the microbiota within the upper respiratory tract to the immune perturbations associated with prolonged exercise is currently unclear. The most popular parameter of mucosal immunity that has been investigated in an acute exercise setting has been salivary SIgA due to the notion that individuals who suffer from IgA deficiency contract URTI regularly (Gleeson and Pyne, 2000). Physiological changes (e.g. nervous stimulation, dehydration) during exercise can influence the secretion of saliva and its protein components (Bishop and Gleeson, 2009; Walsh et al., 2004). The salivary glands are innervated by both the parasympathetic and sympathetic nervous system, with changes in stimulation of either of these having an influence on the volume, viscosity, protein and mucin concentration (Aps and Martens, 2005). Parasympathetic nervous stimulation via vasodilation of the salivary glands is believed to trigger a high volume of watery saliva, low in protein concentration (Bishop and Gleeson, 2009). On the other hand, sympathetic stimulation produces salivary secretions which are low in volume but high in protein that is primarily due to enhanced active transport of proteins from salivary cells (Proctor and Carpenter, 2007). Thus, similar to the other discussed immune parameters, salivary SIgA concentration is susceptible to effects (sympathetic and parasympathetic responses) of exercise intensity and duration (Walsh et al., 2011b).

The following discussion will consider those studies which have used the most reproducible collection method, unstimulated whole saliva collection (also termed passive drool), due to the potential for stimulated saliva flow (e.g. chewing) and other

collection methods (e.g. swabs) to preferentially induce secretion from certain glands and/or influence saliva composition once secreted (Allgrove et al., 2013; Beltzer et al., 2010; Bishop and Gleeson, 2009; Granger et al., 2012; Harmon et al., 2007; Navazesh and Christensen, 1982; Navazesh, 1993; Proctor and Carpenter, 2001).

Although there is inconsistency in study design within the literature to draw a definitive conclusion, Walsh et al. (2011b) suggested that SIgA concentration in saliva generally decreases (e.g. Nieman et al., 2002a; 2003; Palmer et al., 2003; Tomasi, et al., 1982) or remains unchanged (e.g. MacKinnon and Hooper, 1994;; Sari-Sarraf et al., 2006) following prolonged exercise (≥ 1.5 h at 50-75% maximum oxygen uptake, $\dot{V}O_2$max). It seems that the combination of high-intensity and exercise of a long duration has the most significant impact (i.e. depressive) on salivary SIgA concentration (Mackinnon, 1996; Nieman et al., 2002a). The discrepancies in the literature are also believed to be partly due to the way in which salivary SIgA concentration is expressed relative to the exercise-induced changes in physiological responses (Walsh et al., 2002). In an attempt to account for such changes, salivary SIgA concentration has been expressed as a secretion rate or relative to total salivary protein/albumin/osmolality (Gleeson, 2000), but this makes the comparison between studies difficult (Bishop and Gleeson, 2009).

The secretion rate or expression relative to saliva osmolality are preferred over measures of SIgA as ratio to total protein (Blannin et al., 1998). One reason for this is that other salivary proteins (e.g. amylase) are known to increase with exercise without a change in SIgA (Walsh, 1999). Furthermore, expression as a secretion rate may better reflect the amount of available SIgA on the mucosal surface (MacKinnon et al., 1991). In contrast, it has been argued that salivary SIgA concentration is of

greater significance as secretion rate may only provide an explanation to how salivary flow rate has changed (Bishop and Gleeson, 2009). Having said that, salivary SIgA when expressed as a secretion rate has been found to be the best predictor of URI incidence in athletes following a 160 km race (Nieman et al., 2003) or during intense training and competitive phases (Fahlman and Engels, 2005). Additionally, given that the majority of saliva is water, expression relative to secretion rate also accounts for the concentrating effect of other salivary components following any dehydration (Bishop et al., 2000; Oliver et al., 2007). As saliva osmolality reflects the inorganic electrolyte concentration (rather than protein content) and hence falls in proportion with decreases in flow rate, SIgA:osmolality provides an alternative method (Bishop and Gleeson, 2009; Blannin et al., 1998).

Although IgA is the dominant Ig in mucosal secretions, some have also attempted to identify changes in salivary IgG and IgM concentrations following exercise. Limited findings suggest IgG remains unchanged but IgM parallels decreases in salivary SIgA, highlighting the potential effects that acute strenuous exercise has on salivary immune parameters (Bishop and Gleeson, 2009; Gleeson and Pyne, 2000). Another aspect of mucosal immunity which has received little attention to date is the responses of AMPs to exercise (Walsh et al., 2011b). Despite the interest of the dentistry field in the role of AMPs in oral health and infection, (Dale et al., 2006; Putsep et al., 2002; Tanida et al., 2003; Tao et al., 2005), the relationship of AMPs with exercise-induced immune dysfunction is yet to be explored conclusively (Davison et al., 2009).

To date, the few investigations that have been conducted suggest in accordance with other immunological measures, responses of AMPs may be dependent on the

intensity and duration of the exercise bout (Hoffman-Goetz and Pedersen, 1994). A 2 h cycling bout at ~ 65% $\dot{V}O_2$max resulted in a significant decrease in salivary lysozyme (sLys) concentration, sLys secretion rate and sLys:osmolality which recovered after 1 h of recovery (Davison and Diment, 2010). In contrast, expressions of other AMPs (LL37 and defensins: human neutrophil peptide 1-3) following similar stressors (2.5 h at ~ 60% $\dot{V}O_2$max) have been shown to significantly increase immediately post-exercise. In the recovery period (0-1.5 h post) following a 50 km mountain trail race (mean running time ~ 8 h) there was a significant decrease in salivary lactoferrin (sLac) concentration and non-significant decreases in sLac and sLys secretion rate (Gillum et al., 2013).

The mechanism behind the effects of prolonged exercise on salivary parameters (AMPs, SIgA) remains unclear (Walsh et al., 2011b). The flow rate of saliva is considered to be the major source of variation in concentration of mucosal parameters (Gleeson and Pyne, 2000). In general, saliva flow rate decreases in response to a prolonged exercise bout (Bishop et al., 2000; Walsh et al., 1999). Decreases have been attributed to a withdrawal of parasympathetic stimulation rather than sympathetic-induced vasoconstriction of salivary glands (Bishop and Gleeson, 2009). Parasympathetic withdrawal associated with sensations of a dry mouth in response to other acute stressors (e.g. psychological) supports such proposals as does the lack of effect on saliva flow rate with interventions aimed to increase sympathetic stimulation (Bishop et al., 2006; Bosch et al., 2002)

A crucial determinant of SIgA release into saliva is the presence of pIgR to permit transport across the epithelial layer (Bosch et al., 2002). Evidence from animal studies suggest that increased mobilisation of pIgR occurs only above a certain

threshold of increased sympathetic stimulation (Proctor et al., 2003). This may explain why a brief bout of high-intensity exercise leads to increases in salivary SIgA (e.g. Davison, 2011). However, this does not explain the decrease found with prolonged exercise bouts (Bishop and Gleeson, 2009). It has been speculated that this nervous stimulation over a longer period (i.e. prolonged exercise) may deplete the available IgA (Allgrove et al., 2008; Proctor et al., 2003) or there may be a further threshold (duration) where pIgR mobilisation is down-regulated (Walsh et al., 2011b).

As brief bouts of maximal effort exercise results in increased sLac and sLys, it is reasonable to suggest that mobilisation of AMPs during exercise is also influenced by sympathetic stimulation in relation to certain thresholds (Allgrove et al., 2008; Usui et al., 2011; West et al., 2010). Alternatively, the reductions in sLys following prolonged exercise may be related to an increased stimulation of the HPA axis (West et al., 2010). However, investigations thus far have not found a cause-and-effect relationship, rather only reporting an association between increases in salivary cortisol and reduced sLys (Perera at al., 1997). It is worthy to note that the source of sLac and sLys (phagocytes, epithelial cells) is different to SIgA. Therefore, it is possible that increases in AMPs during high-intensity exercise may be as a result of the effects of hyperventilation (e.g. drying of the mucosal surfaces) given that some of these changes are lost or reduced when expressed relative to osmolality or as a secretion rate (Davison, 2011). Additionally, the exercise-induced damage to the epithelial layer could induce an inflammatory response whereby epithelial cells increase release of AMPs and other sources of production of AMPs are recruited (e.g. neutrophils) (West et al., 2010). The relative contribution of AMPs from each of these sources to saliva at both rest and following exercise is unclear. However, it is reasonable to suggest that an airway inflammatory response partly accounts for the

increases in AMPs identified immediately following prolonged exercise (Davison et al., 2009).

Certain AMPs (α defensins) have been suggested to make up 50% of the protein found in neutrophil azurophilic granules (Radek and Gallo, 2007). Muns (1994) demonstrated a significant (twofold) increase in the neutrophil count of mucosal secretions (nasal lavage fluid) following a prolonged exercise bout. Although neutrophils continually migrate into saliva from the circulation via gingival crevices (Bender et al., 2006; Lukac et al., 2003), the extent of neutrophila that occurs in the circulation following exercise may lead to the presence of neutrophils and their contents in saliva (e.g. AMPs) being substantially increased (Davison et al., 2009). Increased levels of AMPs (e.g. α defensins) have been observed in other body fluids (e.g. plasma) following a circulating neutrophila (Shiomi et al., 1993). The variation in the responses of different AMPs to prolonged exercise (Davison et al., 2009; Davison and Diment, 2010), therefore, may be due to the changes in the maturity of circulating neutrophils (as expressions of AMPs can vary throughout the maturation of the neutrophil in the bone marrow) or decreases in some AMPs may merely reflect neutrophils undergoing a refractory period post-exercise (Borregard and Cowland, 1997; Cowland et al., 1995; Gullberg et al., 1997; Nagaoka et al., 1997, 1998, 2000; Peake, 2002; Sorensen et al., 1997) .

1.3.5 Exercise training and immune function

Based on the evidence presented to date regarding acute prolonged exercise, it may be expected that the highly trained demonstrate lower immune function at rest compared to non-exercising controls. However, numerous cross-sectional studies in an attempt to discern such differences have found that when measures of immune

function are gathered in a ‘resting state’ (at least 24 h following the previous bout) there seems to be very little difference between athletes and controls (Gleeson, 2007). A review of early investigations in the area (Nieman, 2000) suggested even when significant immune perturbations had been observed in athletes participating in strenuous exercise, investigators had limited success in identifying such measures that influence alteration in rates of URI (Gleeson et al., 1999; Mackinnon et al., 1991; Nieman et al., 1990b, 1998, 2000).

Despite the large inter-individual variation in concentration of salivary SIgA, (Walsh et al., 2011b) some of the early work (salivary SIgA responses to training) reported lower concentrations in endurance athletes compared to sedentary counterparts (Tomasi et al., 1982). In contrast, most of the recent investigations support evidence of other parameters by showing salivary SIgA to be broadly similar in the two populations (Bishop and Gleeson, 2009; Gleeson and Pyne, 2000). Conversely, recent findings in elite rowers had 50-60% lower sLac concentration than controls at the start and mid-way through a 5 month training period (West et al., 2010), suggesting investigations of the mucosal immune compartment in a ‘resting state’ between these populations requires further attention. Inverse relationships between salivary SIgA concentration/secretion and URI incidence in some studies of athletes undergoing periods of intensive training (i.e. athletes not necessarily in ‘resting state’) (Fahlman and Engels, 2005; Gleeson et al., 1999; Neville et al., 2008) further supports the need for longitudinal research on mucosal immunity with large athletic populations (Nieman, 2001). The common observations of this mucosal parameter within intensive training studies represents a narrow range that have established a link between an immune measure and URI in athletes (Gleeson, 2007; Walsh et al., 2011b).

Given the complexity of the immune system, it is unlikely that salivary SIgA alone would explain URI risk for all athletes, thus other factors in combination with the immune measure need to be determined (Nieman and Bishop, 2006). Investigators have begun attempts to address this by examining differences between illness prone and healthy athletes with early evidence suggesting that the blood obtained from the illness prone have greater *ex vivo* production of anti-inflammatory cytokines (e.g. IL-4 and IL-10) in response to multiantigen challenge, indicative of a down regulation of cell-mediated immunity (Gleeson and Bishop, 2013; Gleeson et al., 2012b).

Multifactorial mechanisms beyond salivary SIgA alone in URI incidence become more apparent when athletes intensify their training over a short period of time (Gleeson, 2007). Indeed, 1–3 weeks of intensified training has been shown to induce marked reductions in neutrophil and monocyte function, lymphocyte proliferation and the circulating number of T cells (Lancaster et al., 2003, 2004; Robson et al., 1999a; Verde et al., 1992). In addition to intensive experimental protocols, longitudinal monitoring over competitive seasons showed that team sports (rugby) and endurance athletes (cyclists) are sensitive to the intensive training periods with decreases in T cell counts, sLys, neutrophil oxidative burst and IL-2 production (Baj et al., 1994; Cunniffe et al., 2011).

These findings suggest that there is a cumulative effect of repeated bouts of strenuous exercise due to an inadequate recovery time for the immune system (Papacosta and Gleeson, 2013). Furthermore, it may be that responses to acute prolonged exercise are more clinically relevant than resting immunity in athletes where the immediate recovery period represents the ‘open window’ when athletes are most vulnerable to infection (Abbasi et al., 2013; Nieman et al., 1990b, 1994;

Pedersen and Bruunsgaard, 1995). Despite the lack of clear difference in measures at rest, as athletes are exposed to more frequent acute bouts (‘open window’) of immunodepression, the overall risk may be considered greater than non-exercising controls. The presence of any other risk factors (e.g. dietary energy deficiency, sleep disturbance, psychological stress) will have additive effects on the transient immunoendocrine responses and increase the risk of illness following prolonged exercise (1.5 h at 55–75% $\dot{V}O_2$max), as seen in military studies (Gleeson, 2007). Although underlying causes of URI remain uncertain, decrements in performance as a result of URI have been reported (Pyne et al., 2001, 2005; Reid et al., 2004). The monitoring of the risk status of an athlete is paramount in order to determine the appropriate preventive or therapeutic interventions required during stages of strenuous training/competition (Pyne et al., 2000, 2001).

1.4 Immunonutrition and bovine colostrum

Numerous factors are known to influence the immune response and infection risk following prolonged/strenuous exercise (Walsh and Gleeson, 2013). Modulation of the immune response to prolonged exercise with nutrition is an active and ongoing area of research (Nieman and Bishop, 2006). Macronutrients play an integral role in immune cell metabolism and protein synthesis while micronutrients are important in immune cell replication and antioxidant defences (Bishop et al., 1999). It is well accepted that both macronutrient and micronutrient deficiencies impair immune function, the magnitude of which is determined by the severity or duration of the deficiency (Gleeson, 2006a). Such inadequate nutrient availability can have direct or indirect effects on the immune system (Gleeson and Bishop, 2000). Although correcting for a nutritional deficiency usually restores immune function and

resistance to infection (Calder and Kew, 2002), physiological stress placed on the immune system by exercise and/or presence of infection may require additional immunonutrition support (Calder and Jackson, 2000; Nieman, 2008).

As important as direct effects of nutrients (e.g. fuel source for cells) are on immune activity, it is considered that most of the immune perturbations following prolonged exercise are mediated by indirect effects such as changes in immunoregulatory neuroendocrine hormones (e.g. adrenaline, cortisol, noradrenaline) that occur in response to low nutrient availability (e.g. low blood glucose) (Akerstorm and Pedersen, 2007; Gleeson et al., 2004). For this reason additional carbohydrate during prolonged exercise (which can maintain blood glucose) can reduce much of the physiological stress (stress hormone responses) placed on the immune system (Nieman and Bishop, 2006). Although prolonged exercise can also reduce circulating concentrations of certain amino acids and induce a state of oxidative stress, nutritional agents aimed to attenuate these responses (e.g. glutamine, vitamin C) have shown inconsistent effects in regards to limiting exercise-induced immune dysfunction, particularly if dietary intake is adequate (Hiscock and Pedersen, 2002 Nieman et al., 1997a, 2002b). Indeed, with the exception of carbohydrate beverages, many immunonutrition support strategies during exercise have been shown to be ineffective (Gunzer et al., 2012).

However, use of carbohydrate supplementation during exercise in some studies has also shown minimal effects on the exercise-induced dysfunction of innate (e.g. neutrophil degranulation, NKCA) or mucosal parameters of the immune system (salivary SIgA and AMPs) (Bishop et al., 2000; 2001a, 2001b; Nieman et al., 1997b; Walsh et al., 2011a). Therefore, it seems that carbohydrate feeding may act as a

partial countermeasure to exercise-induced immunodepression (e.g. cytokine release, leukocyte trafficking) (Nieman, 2008). This has led to suggestions that a cocktail strategy which combines carbohydrate with a mixture of advanced supplements will be more effective by targeting the diverse complexity of the immune system (Bakker et al., 2010; Nieman et al., 2009; Walsh et al., 2011a). Furthermore, there is an increased prevalence of undertaking some training in a low carbohydrate state for a proposed enhancement in adaptation from exercise bouts (Philp et al., 2012). Although the efficacy of training in a low carbohydrate state is a whole other area (not for discussion here) with current debate on the effectiveness of this method in practice (i.e. to actually increase performance more), it is nonetheless becoming a more common practice for some athletes. It is essential that other nutritional strategies are sought to prevent the expected immune disturbance with such strategies (Bishop et al., 2001b; 2001c), particularly when athletes may also exercise later on in the day (Ronsen et al., 2001a, 2001b). It is also worthy to note that even with the ability of carbohydrate to attenuate the stress hormone response to prolonged exercise, there is limited evidence to suggest that these effects translate into a reduced incidence of URI (Gleeson et al., 2004; Nieman et al., 2002a). As such, it has been proposed that nutritional supplements which target the non-specific first line of defence (innate immunity) compared to the slower responding adaptive immune system may provide more effective sources of immunosurveillance and resistance to a wide range of pathogens (Walsh et al., 2011a)

Bovine colostrum (COL) is the initial milk produced by a cow in the first few days following parturition. In addition to a different composition of macronutrients (higher percentage of protein, lower percentage of lactose and fat) compared to mature milk (Ontsouka et al., 2003) COL is richer in antimicrobial, growth and immune factors

(Uruakpa et al., 2002). In fact, the bioactivity of COL is at its greatest in the first milkings with the concentrations of such components decreasing over the subsequent days (Ginjala and Pakanen, 1998; Hagiwara et al., 2000; Korhonen et al., 2000; Mandalapu et al., 1995). The collection of COL closer to the first day of calving is not only essential for the calf but along with processing methods is a crucial determinant of quality of COL as a nutraceutical for other species (e.g. humans) (Shing et al., 2009a). It is proposed that the greater concentration of bioactive peptides (compared to other mechanisms in the neonate e.g. Ig) at this stage are the important modulatory components of COL for human immunity (Davison, 2013).

For the newborn calf, the delivery of COL is crucial for provision of energy and growth factors for the development and maturation of the gastrointestinal tract (Blattler et al., 2001; Rawal et al., 2008). COL also manifests antioxidant properties (e.g. Vitamin A and E) that provide crucial protection for the calf against the sudden increase in oxygenation and hence oxidative stress in the external compared to intrauterine environment (Przybylska et al., 2007). Unlike humans, *in utero* antibody transfer during foetal life is limited (Coons et al., 2012). Therefore, the antimicrobial and immune components of COL play important roles in promoting resistance against pathogens and ensuring the survival and health of the newborn (Brinkworth and Buckley, 2003). Lactoferrin and lactoperoxidase are the dominant non-specific antibacterial and antiviral components of COL that protect the calf from numerous infectious microorganisms (Thapa, 2005; van Hooijdonk et al., 2000). Other components such as oligosaccharides and glycoconjugates are also important bioactive molecules that provide protection by inhibiting pathogen adhesion to epithelial surfaces (Gopal and Gill, 2000). However, it is the rich source of acquired

immunity (IgA, IgG and IgM) in COL that has a well-recognised role of laying the foundations for the neonate immune system through the non-selective macromolecular transport of passive immunity following birth (Korhonen et al., 2000).

The accumulation of Ig in COL from maternal circulation occurs several weeks prior to parturition and thus reflects the antigen exposure of the mother (Gapper et al., 2007; Kelly, 2003; van Hooijdonk et al., 2000). This has evolved into the use of selective inoculation of cows in order to raise specific Ig in COL which provides immunisation for the calf against certain enteric infections (Solomons, 2002). Such hyperimmune colostrum has also been investigated as a potential strategy for the prevention and treatment of gastrointestinal diseases in humans with beneficial effects reported against rotavirus, cryptosporidiosis and other opportunistic pathogens which prevail under situations of immune deficiency (e.g. HIV) (Floren et al., 2006; Hurley and Theil, 2011; Kelly, 2003).

COL (i.e. non-hyper immune) still contains amounts of Ig which are up to a 100 times greater in concentration than mature milk that can aid in neutralisation or clearance of pathogens (Mach and Pahud, 1971). However, it has been suggested that collection needs to be refined to less than 6 h post-partum to possess IgG concentrations which offer optimal protection in other species (i.e. humans) (Gapper et al., 2007). For this reason, it has been suggested that the level of IgG (most abundant colostral Ig) is a determinant of COL quality (Gulliksen et al., 2008).

Investigations of the bioavailability of bioactive components *in vivo* following the oral digestion of COL in humans is an underexplored field whereby most studies to date have speculated on the effects on humans through activity of isolated peptides *in vitro* (Ross et al., 2013). However, the bioavailability of COL in the adult human

intestinal tract can be based on the few studies of milk protein digestion (Boutrou et al. 2013; Chabance et al., 1998; Mahe et al., 1991), which demonstrate that bioactive peptides can reach concentrations in the intestine that are likely to exert biological and/or synergistic activity locally or in the blood stream upon absorption (Boutrou et al. 2013). The casein fraction of milk protein precipitates within the stomach and leaves as degraded products resulting in a slower gastric transit time than the whey fraction (6 h vs. < 3 h) which is soluble in the stomach and proteins leave partially intact (Boutrou et al. 2013). Due to the presence of other immune regulating components (cytokines, growth factors) in COL which act as messengers within the human immune system, COL has been considered a 'universal donor' to humans (Pakkanen and Aalto, 1997). Indeed, although sharing a homologous composition, the concentrations of immune factors in COL are in vastly greater concentrations than human colostrum (Shing et al., 2009a). This has led to suggestions that COL could enhance human immune function and hence afford prophylaxis of infections at sites other than the gastrointestinal system, with the area of URI receiving much interest (Brinkworth and Buckley, 2003; Shing et al., 2009a).

1.4.1 Bovine colostrum and upper respiratory illness

Historically, COL has been considered to have many purported benefits to humans (Thapa, 2005). This is largely due to its nutraceutical properties showing similarity in structure and function across species (Pandey et al., 2011). Prior to the antibiotic era, COL was used in the treatment of bacterial infections (Thapa, 2005). More recently, however, COL has been advocated as a nutritional supplement for children and adults to prevent, or treat, URI (summarised in Table 1.1). As shown in Table 1.1, these studies have used a range of methods to report and/or monitor URI. For clarity, as none of these studies used serological analysis to identify a causative pathogen, all episodes defined within the studies will be considered as a URI.

Brinkworth and Buckley (2003) were the first to investigate the effects of COL on URI in a double-blind placebo (PLA) controlled manner. This involved the retrospective analysis of log books of self-reported URI obtained from previous studies which investigated the effects of COL on physiological responses in physically active males (Brinkworth and Scamell, 2000; Buckley et al., 2001, 2002). Following randomised allocation, it was shown that there was a significantly lower proportion of participants who reported URI episodes (32%) in the COL group (60 $g \cdot day^{-1}$) compared to the PLA group (48%). This effect was observed when comparing both groups in the final 7 weeks of the supplementation period. The total duration of supplementation was 8 weeks but authors chose to analyse the first week of supplementation separately to avoid symptoms arising from illness prior to the start of study. There were no differences between groups (COL = 11%, PLA = 5%) in the proportion of participants who reported URI within this first week. Further investigation of participants who only reported URI episodes during the final 7 weeks

demonstrated no difference in mean number of episodes between COL (mean ± standard deviation, 1.2 ± 0.4) and PLA (1.1 ± 0.3). Additionally, there was no difference in the duration of URI episodes between groups. A limitation of this study was the lack of data from the individual studies. These studies included participants who were involved in endurance and resistance training, hence it is difficult to ascertain whether the effects of COL on URI are universal or more apparent in those who partake in endurance exercise (i.e. experience the most immunodepression). Nevertheless, these findings suggest a role of COL supplementation in reducing the proportion of human participants reporting URI. The lack of effect of COL on the mean number of episodes in those who did report URI was not surprising given the short duration of the intervention.

Crooks et al. (2006) followed this with a prospective investigation of the effects of COL on URI in a homogenous sample (distance runners). Although there was a lower mean number of URI days (5) in a group who received 12 weeks of COL supplementation (10 $g \cdot day^{-1}$) compared to PLA group (8), this difference was not significant. Additionally, there was no difference between the mean number of URI episodes in the groups. Such findings did not support Brinkworth and Buckley (2003), however, this could be explained with the six fold difference in dose of COL and hence potential bioactive components. When Shing et al. (2007) investigated a shorter duration (8 weeks) but similar daily dosage (10 $g \cdot day^{-1}$) of COL in highly trained cyclists there was a trend for a lower number of reported URI (using the validated Wisconsin Upper Respiratory Symptom Survey) compared to the PLA group during periods of high-intensity training. Recently, Shing et al. (2013) also investigated the effects of 10 $g \cdot day^{-1}$ of COL for 8 weeks prior to and during a 5 day competitive cycle race on susceptibility to URI. There were tendencies for a lower

number of reports and days of URI in the COL group compared to PLA, where it was suggested that the statistical significance of such outcomes may have been limited by the small sample size of the pilot study. In another cohort of endurance athletes who may be considered most at risk of URI due to intensive training (elite swimmers), Crooks et al. (2010) found trends for a lower proportion of participants reporting URI in a COL group (10 weeks supplementation of 20 $g.day^{-1}$) and lower number of URI days in these participants than PLA swimmers. There were no differences in URI between a non-exercising COL and PLA groups.

Such evidence may suggest that the effects of COL on URI become more apparent when there is a greater scope for intervention (i.e. periods of immunodepression). Although the study of Crooks et al. (2006) would not support this hypothesis, it would provide an explanation behind the efficacy of COL intervention studies involving IgA deficient children (Patiroglu and Kondolot, 2013) or children who suffer from recurrent infections (Patel and Rana, 2006). Patel and Rana (2006) found that a daily dose of 3 g of COL reduced the number of URI episodes at 4 weeks (73%), 8 weeks (83%) and 12 weeks (91%) supplementation compared to baseline (6 months prior to enrolment for study). Compared to baseline there were also significant reductions in the number of children who reported URI at 4 weeks (21%), 8 weeks (36%), and 12 weeks (64%) following COL supplementation.

The study of Patel and Rana (2006), however, was confounded by a number of limitations. As noted by Menon et al. (2010), the baseline URI data should have been based on the mean reports at monthly intervals not the whole 6 months prior to enrolment to the study. The comparisons completed in this study provides an inaccurate and biased interpretation of the effects of COL as monthly episodes of

URI would likely be lower than the incidence in a 6 month period, particularly in a cohort of children who suffer from recurrent infections. Although the study states a high involvement of paediatricians within the study, there is no detail on how URI episodes were diagnosed. The study reports that the number of episodes was assessed at the end of each 4 weeks with no indication of daily reporting of symptoms which significantly undermines the validity of the data. This is highlighted further by the lack of PLA controlled comparison and open label design of this study given that this cohort would require interpretation from others (i.e. parents) prior to observation by the paediatrician.

In a separate cohort of immune-deficient children, Patiroglu and Kondolot (2013) randomly allocated 1 week of COL (3 sucking tablets daily, each containing 14 mg COL and 2.2 mg of lysozyme) or PLA (sucking tablet prepared as 'candy') treatment upon presentation of symptoms in the upper respiratory tract at the hospital. Although there were no differences in duration of symptoms, there was a significantly lower severity of illness in the COL group. However, this study is confounded by symptom severity being reported by the mothers of the children and that COL tablets also contained lysozyme which alone could be suggested to have a preventive effect on symptoms.

In addition, to the above mentioned specific effects of COL on URI, it is worthy to note that that COL has been shown to have prophylactic effect against influenza in both healthy participants and high-risk cardiovascular patients (Cesarone et al., 2007). Although it was not clear how participants were blinded or allocated into groups, the comparison of healthy participants included a COL group (8 weeks of a daily 400 mg dose as a chewable tablet), COL and influenza vaccination (8 weeks of

a daily 400 mg dose within 2 weeks of vaccination), vaccination or a control group (receiving no vaccination or COL). It was found that within the 8 week supplementation period and in the 4 weeks following all interventions, both COL groups suffered fewer episodes of influenza. The COL groups were comparable in terms of influenza episodes, suggesting no additive effect of vaccination. This intervention was followed up in the second part of the study with high risk cardiovascular patients. Given the health status of these patients, investigators deemed it was not plausible to have a group who had no prophylaxis (i.e. no control group). Nevertheless, in this cohort it was again demonstrated that the group who received vaccination only had significantly greater number of influenza related complications than both COL groups.

The findings of Cesarone et al. (2007) along with those summarised in Table 1.1, suggest that there is a role for COL in preventing URI. The evidence to date, however, is based on a number of studies which have examined a range of supplementation periods (duration and dosage), populations (children and adults) and study design (lacking PLA groups or use of similar URI monitoring). It is reasonable to suggest, however, that the effects of COL are more pronounced in those populations who are in a state of immune deficiency or suffer from greater periods of transient exercise-induced immune dysfunction. It can therefore be hypothesised that COL may contribute as a source of additional immune components in these situations and/or stimulate host immunity. For this reason, numerous investigators (some already mentioned above) have attempted to identify whether COL and/or its components influence human immune function (Jensen et al., 2012).

Table 1.1 Summary of studies that have examined the effect of COL on URI

Reference	Design	Population	Supplementation	URI monitoring	URI criteria	Findings
Brinkworth and Buckley, 2003	DB, PC, R	174 Physically active males aged 18-35 years	8 weeks of 60 g.day^{-1} of concentrated COL (n = 93) or whey protein (n = 81)	Daily self-reports of symptoms	Coded on criteria of Centre for Disease control; URI: ≥ 2 consecutive days of coded cold/flu symptoms	Lower proportion of participants reported URI in COL over the 8 weeks ($p < 0.05$). No difference in number of and duration of episodes ($p > 0.05$).
Crooks et al., 2006	DB, PC, R	35 distance runners (15 females, 20 males) aged 35-58 years	12 weeks of 12 g.day^{-1} COL (n = 18) compared to isomacronutrient skimmed milk (n = 17)	Daily self-reports of symptoms	2 consecutive days of cold/flu symptoms	No difference in mean number of URI episodes ($p > 0.05$). No effect on total number of URI days ($p > 0.05$).
Patel and Rana, 2006	OL, NC	551 children aged 1-8 years with recurrent URTI (> 6 episodes in previous 6 months)	12 weeks of 3 g.day^{-1} of COL	Follow up with Paediatrician at end of each 4 weeks	Not specified	Lower number of URI episodes and patients reporting URTI at 4 weeks, 8 weeks and 12 weeks compared to baseline (previous 6 months) in all age groups ($p < 0.05$).
Shing et al., 2007	DB, PC, R	29 highly trained cyclists	8 weeks of 10 g.day-^{1} of concentrated COL (n = 14) or whey protein (n = 15)	Daily self-reports of symptoms (Wisconsin Upper Respiratory Symptom Survey)	≥ 2 upper respiratory symptoms	Trend for lower number of self-reported illnesses in COL group ($p = 0.06$) No difference in duration of illness ($p > 0.05$)
Crooks et al., 2010	DB, PC, R	25 (12 male, 13 female) elite swimmers, 28 aged-matched non-exercising CON	10 weeks of 20 g.day^{-1} of low protein COL (12 swimmers, 16 CON) or isomacronutrient skimmed milk (13 swimmers, 12 CON)[1]	Daily self-reports of symptoms	2 consecutive days of cold/flu symptoms	Trend for lower mean number of URI days reported per participant in COL swimmers compared to PLA swimmers ($p = 0.08$). Trend for lower proportion of subjects reporting URI in COL group weeks 1-4 ($p = 0.06$). No difference between COL and PLA CON ($p > 0.05$.)
Patiroglu and Kondolot, 2013	DB, PC, R	31 (18 male, 13 female) IgA-deficient children aged 5-17 years with acute respiratory symptoms	1 week, 3 sucking tablets daily of COL(14 mg and 2.2mg lysozyme) (n = 16) or matched PLA (n = 15) tablet (not specified)	Daily 10-point symptom severity scale completed by Mother	Diagnosis of URI by paediatrician for study inclusion; Daily 10-point symptom severity scale completed by Mother	Lower severity score after COL ($p < 0.05$). No difference in duration of symptoms ($p > 0.05$).
Shing et al., 2013	DB, PC, R, P	16 highly trained cyclists	8 weeks and 5 days of 10 g.day-^{1} of concentrated COL (n = 8) or whey protein (n = 8)	Daily self-reports of symptoms (Wisconsin Upper Respiratory Symptom Survey)	≥ 2 upper respiratory symptoms	Mean number of URI days tended (non-significant, $p > 0.05$) to be higher in PLA group. No difference in number of reports URI (COL = 2 versus PLA = 4, $p > 0.05$).

CON- control; DB – double blind; NC; non-comparative; OL- open label; P – pilot study, PC – placebo controlled; R- randomised;

1.4.2 Bovine colostrum and cellular innate immunity

The use of COL as a nutraceutical is an expanding niche (Severin and Wenshui, 2005), with research being performed on whole COL, and on isolated COL compounds (e.g. whey, casein, lower molecular weight fractions) (Jensen et al., 2012). Although the composition of COL will rapidly change over the first few days of lactation, many animal studies have shown that many of its components act in an adjuvant manner on the immune system in a range of species (Pandey et al., 2011). Even though findings from animal models can be considered to have questionable relevance and transferability to humans (Vukmanovic-Stejic et al., 2006), it is worthy to note that they have played an important role in directing investigations of COL within human *in vitro* culture (Table 1.2). Due to the importance of innate immunity in resisting infection of the mammary gland of dairy cows in the periparturient period (Roth et al., 2001), *in vitro* studies have investigated how COL may affect capacity of innate cells. As impaired neutrophil function of the cow is common during this period and COL contains a vast amount of cytokines which regulate neutrophil function it is a prudent area of research (Dosogne et al., 1999; Kerhli et al., 1989).

In vitro treatment of PMN cells (i.e. neutrophils) obtained from cattle with COL or milk components shows improvement in chemotaxis, phagocytosis and killing capacity (Roth et al., 2001; Sugisawa et al., 2001, 2002, 2003). Such evidence may suggest that the exposure of neutrophils to priming cytokines such as IL-1, IL-6 and TNFα within COL amplifies the response to subsequent activation *in vitro* (Goto et al., 1997; Hagiwara et al., 2000; Sugisawa et al., 2002). Although the effect of priming depends on the agent responsible it may involve increased expression/affinity of surface receptors, modulation of secondary messengers (e.g.

calcium) or mobilisation of intracellular components (e.g. NADPH complex) (Atkinson et al., 1988a, 1988b; Peake, 2002, Rusu et al., 2009; Tennenberg and Solomkin, 1990). However, such hypothesis may be upheld by these priming effects of COL also being observed with investigations of bovine milk (lower concentrations of cytokines) (Sugisawa et al., 2002). As the greater concentrations of COL (50%) incubated with PMN cells inhibited rather than promoted oxidative burst responses in the study of Sugisawa et al. (2002), it was suggested that within *in vitro* culture the antioxidant activity of COL may mask the priming activity. Dose dependent increases of PMN phagocytosis have only been apparent up to a pre-treatment of 25% COL concentration when compared to milk and bovine serum (Sugisawa et al., 2001). Similar to IgG levels the greatest promotion of phagocytosis was observed in COL obtained immediately after parturition with such activity declining in COL obtained at a later date but at a slower rate than IgG levels. Sugisawa et al. (2002) suggested that the enhancement of neutrophil functions were due to priming of responses rather than an opsonisation mechanism as there was no enhancement of oxidative burst when *Staphylococcus aureus* was opsonised by COL.

Sugisawa et al. (2003) further investigated by fractionating both COL and bovine milk and exposed neutrophils from cows to identify any differences in oxidative burst activity. *In vitro* culture demonstrated that only a low molecular weight fraction (< 10 kDa) of COL and milk had a priming effect on oxidative burst activity to neutrophil activation by *Staphylococcus aureus*. It was suggested that that beneficial effects on neutrophil function may not be related to the previously mentioned pro-inflammatory cytokines where all (except for IL-8) have molecular weights between 10-30 kDa (Cerretti et al., 1986; Cludts et al., 1993; Ebrahimi et al., 1995; Maliszewski et al., 1988; Swain et al., 2002), but smaller constituents (< 10 kDa) of COL that are not

well understood (e.g. proteose peptones) (Merin et al., 2001; Sugisawa et al., 2003). Of interest, given that COL was diluted 10 fold compared to bovine milk, these constituents like many other bioactive peptides are present in greater quantity in COL (as reported by Sugisawa et al. (2001)). The priming effect of this low molecular weight fraction of COL was deactivated by heat exposure and not effective prior to activation with all subsequent neutrophil activation agonists (e.g. PMA).

The priming effects of bovine derived products on neutrophil function have also been demonstrated with human *in vitro* cell culture studies (Rusu et al., 2009, 2010). Compared to a control medium, prior incubation with bovine whey protein extract was shown to significantly amplify chemotactic and phagocytic responses, generation of superoxide ions and release of primary granules content of neutrophils upon stimulation by fMLP (Rusu et al., 2009). This study suggested that priming effects occurred through the components, β-lactogloulin and α-lactalbumin of whey protein extract. The relevance of these priming agents to those reported with COL in animal culture is unclear, but does suggest that COL and bovine milk may play a role in promoting innate immunity in humans. In addition to the ability of whey protein extracts to induce intracellular signalling within neutrophils and translocation of the NADPH-oxidase subunits (e.g. p47phox) to the plasma membrane, it was also shown to induce neutrophil cytokine production (e.g. IL-1 receptor agonist) similar to other priming agents (e.g. GM-CSF) (Malyak et al., 1994; Roberge et al., 1994; Rusu et al., 2010). Direct effects on production of other cytokines (IL-8, IL-6, TNFα) by neutrophils was also observed.

The ability of COL in particular to modulate cytokine production has also been shown in other cell subsets of the immune system. Increasing concentrations of COL, *in*

vitro, have been shown to modulate cytokine production in PBMC from resting healthy humans, including: increased IFN γ, IL-10 and IL-2 production and decreased TNF production in unstimulated samples; decreased TNF and IL-4 production in LPS-stimulated samples; and increased IL-10 production and decreased IFN γ, TNF and IL-4 production in PHA-stimulated samples (Shing et al., 2009b). Although some of these findings suggest an anti-inflammatory profile, following 24 h incubation of PBMC with COL, only IFN γ and IL-2 remained elevated, indicative of a T^h1 cytokine profile.

Effects on PBMC in an unstimulated state (e.g. increase IL-2 production) may provide an explanation to the increase in CD4+ T cell counts found in immunosupressed patients following a period of COL supplementation (Floren et al., 2006). However, as there was no comparable PLA group of immunosupressed patients in this study, the effects observed are similar to those seen with other micronutrient supplementation (Evans et al., 2013) which may suggest that addressing the poor nutritional status associated with the infection of these patients is the important contributing factor. Nevertheless, the findings of Shing et al. (2009b) do propose a role for COL in modulation of cytokine production with particular dominance of a T^h1 cytokine profile. However, this may depend on the stimulus as COL did inhibit T cell proliferation following induction by mitogens (e.g. PHA).This supports the differential effects of COL observed by Biswas et al. (2007) who demonstrated that COL had a slight (non-significant) inhibition of PMBC proliferation but showed a dose-dependent increase in IL-12 which promotes a T^h1 profile during antigen challenge. Although COL enhanced IFN γ production under weak antigenic stimulation, it inhibited IFN γ with strong antigenic stimulation (Biswas et al., 2007).

This biphasic mechanism of COL was also observed in Jenny et al. (2010) where a T^h1 immune response was promoted in unstimulated PBMC but suppressed in mitogen-stimulated PBMC. Furthermore, in a culture of peripheral blood leukocytes obtained following 6 weeks of COL or PLA supplementation, it was found that the COL group had a substantially lower mitogen (concanavalin A)-induced IFNγ production (Appukutty et al., 2011). These inhibitory effects on aspects of T cell proliferation may be supported by a preliminary *in vivo* investigation suggesting that a topical ointment containing COL may alleviate skin lesions of patients suffering from conditions (atopic dermatitis, psoriasis) which are characterised by dermal infiltration of T cells (Pedersen and Hidayat, 2010).

Wolvers et al. (2006) reported no effects of 6 weeks of COL supplementation on mitogen-induced lymphocyte proliferation or DTH response to the CMI Multitest ® containing six previously encountered antigens (Tetanus, Diptheria, *Streptococcus* group C, Candida albicans, Trichophyton mentagrophytes, Proteus mirablis). Although not all data were shown, this study also suggested no effect of COL on granulocyte phagocytosis and oxidative burst. The lack of effect on granulocyte function are not in accordance with *in vitro* studies (Sugisawa et al., 2001, 2002, 2003) but this could be explained by the low daily dose given to participants (1.2 g) which may have been an insufficient dosage for the accumulation of bioactive peptides suggested to affect innate immunity.

Despite previously mentioned benefits towards reduction of URI with low doses of COL (e.g. Cesarone et al., 2007), the majority of these studies have included participants with underlying immune dysfunction which potentially would acquire greater benefits from COL rather than the healthy cohort of Wolvers et al. (2006). It

is reasonable to suggest that for the priming/immune enhancing effects on innate immune cells to take hold systemically, in healthy individuals, the COL must be of a sufficient dose to augment the bioavailability of COL or the effects may be localised to the sites with greatest exposure to the bioactive peptides.

1.4.3 Bovine colostrum and mucosal immunity

COL is a precursor for vast amounts of potentially bioactive peptides that are embedded within the intact structure (Clare and Swaisgood, 2000; Pihlanto and Korhonen, 2003). Although some proteins can pass intact through the neonatal intestine (e.g. Ig) and offer vital protection against pathogens, protease digestion of other proteins leads to peptides becoming bioavailable to have localised effects within the intestinal environment and passage through the GI tract (Jorgensen et al., 2010).

Most of the studies of GALT and COL have involved the use of animal tissue but these may shed light on the potential *in vivo* mechanisms within humans. Yoshioka et al. (2005) demonstrated that T cells (intestinal intraepithelial lymphocytes) of mice following 6 weeks COL supplementation were polarised to the T^h1 type. The reported effects of COL in the GALT were deemed to be direct on the T cells as there were no changes in fecal SIgA or intestinal microflora. Direct effects of COL were also observed in a culture of murine PP cells where there were significant increases in IL-12 and IFN γ (type 1 cytokines) in a dose-dependent manner (Uchida et al., 2011). Similar dose dependent effects of COL were shown within NKCA of PP cells (in the same study) whereby a group of mice who received 400 $mg \cdot g^{-1}$body mass (BM) (6 doses per week for 3 weeks) were not different to a control group but

there was a significantly higher NKCA in mice who received a higher dose of COL (2000 mg·g^{-1}BM). This pattern was replicated in both splenocytes and lung cells.

As the *in vitro* effects of COL on IL-12 and IFN γ were not present in serum following COL supplementation but there were direct effects *in vitro* on IFN γ production in lung cells, it was suggested that augmentation of NKCA in the lungs was due to NK cells activated in PPs (by COL), homing to the spleen and lungs. Such mechanism remains unclear as other investigations involving immunosuppressed mice have shown direct pleiotropic effects on splenocyte cytokine production, with COL supplementation enhancing IL-2, IFN γ, GM-CSF (Wan et al., 2010). Nevertheless, the COL group (2000 mg·g^{-1}BM) in Uchida et al. (2011) showed a lower accumulated symptom rate compared to the PLA group when mice were nasally infected with influenza at the end of the supplementation period. This infection method involved spreading the virus from the nasal cavity to the lower respiratory tract. The above mentioned transfer of innate immunity from intestinal tract to the respiratory tract was advocated to have triggered the amelioration of infection by COL.

Further investigations conducted by Benson et al. (2012) support the hypothesis of local protection at mucosal sites with COL. It was shown that mice that underwent treatment (0.5 and 5.0 mg) with a lactose-reduced, low Ig extract of COL (CLMWF) 30 min prior and 4 h following intranasal *Streptococcus* infection had a lower bacterial load in the lungs compared to a control group. Additionally, in mice treated with similar doses of CLMWF at 24 h and 1 h prior to intranasal influenza infection, CLMWF groups had significantly lower viral loads in the lung than a control group. Both of these infection models involved intranasal and oral treatment with CLMWF, with oral treatment showing the greatest antibacterial and antiviral activity.

Benson et al. (2012) and a companion paper (Jensen et al., 2012), suggest that these effects were likely due to a combination of cellular events that occur at mucosal sites following CLMWF. Benson et al. (2012) found that human PMN cells (rather than murine) treated *in vitro* with CLMWF compared to non-treatment resulted in a greater phagocytosis and chemotaxis in response to fMLP. Jensen et al. (2012) observed an increase in phagocytic activity from baseline in human PMN obtained at 1 h and 2 h following consumption of CLMWF (150 mg) compared to PLA, ran in a counterbalanced fashion (Jensen et al., 2012).

In vitro culture of PBMC cells also involved treatment with COL where it was shown that expression of CD69 was increased by CLMWF and CD25 by COL with these changes being associated with increased cytotoxic activity and proliferation respectively (Benson et al., 2012). Cell surface expression for both CD69 and CD25 was also significantly increased in T cells by CLMWF but not COL. The translation of such effects on human cells to the animal infection models completed in the same studies is difficult but as these studies involved a COL derived product lacking Ig it does suggest the need for studies to investigate whether acute exposure to COL triggers immune priming or enhancing events at local mucosa which may also result in systemic effects.

As there is a major involvement of Igs at all mucosal surfaces, much interest in the effects of COL have been devoted to Ig. Similar to influenza immunisation (Cesarone et al., 2007), humans who were exposed to an oral *Salmonella* vaccine (which mimics enteropathogenic infection) during COL supplementation (100 ml·day^{-1}) showed trends for a greater production of specific IgA (He et al., 2001). Igs are essential mediators of humoral and mucosal immunity but even with the high

endogenous levels of IgG within COL, investigators have failed to see changes in resting circulating levels of IgG or other analogues (IgA, IgE, IgM) in humans following supplementation (Mero et al., 1997, 2002; Shing et al., 2007) which is likely due to the digestion of such components (Davison, 2013).

Given the potential for COL to promote the integrity of the human epithelium (He et al., 2001) and its ability to stimulate GALT in animal models, it would be expected to see benefits on mucosal Ig. As IgA is the most predominant mucosal Ig, most investigators have focused on this parameter (Table 1.3). These have primarily involved an assessment of SIgA in saliva due to the non-invasive nature of sample collection and as production here may be influenced by stimulation of other MALT (PP and tonsils) (Brandtzaeg et al., 1999; Crooks et al., 2006) (summarised in Table 1.3).

COL is a source of transforming growth factor (TGFβ), considered to play a role in mucosal biosynthesis and secretion of IgA (Chen et al., 1990). Mero et al. (1997) found no changes in IgA concentrations in saliva following 8 days of 25 ml or 125 ml COL supplementation compared to PLA. However, when the COL supplementation period is increased (2 weeks) and the dosage is split across the day (4 × 5 g), Mero et al. (2002) found a 33% increase in resting salivary IgA concentration. These findings were confounded with limitations such as the use of a PLA which did not have similar macronutrient content (maltodextrin) and lack of detail in analytic procedures. The methodology is particularly important given that COL contains IgA of bovine origin so analysis of saliva for changes in IgA should be controlled for the human form of Ig. However, in comparison to an isomacronutrient PLA, Crooks et al. (2006) also observed significant increases (79%) in median resting salivary IgA

concentrations from baseline to 12 weeks of COL (12 g·day^{-1}) supplementation in a group of distance runners. Although this result was of statistical significance, investigators did stress that interpretation should be met with caution due to the large variability in IgA levels.

Only one further study of active young adolescents has shown beneficial effects of COL supplementation on resting salivary IgA concentration (Appukutty et al., 2010) whereas many other studies involving exercising (elite swimmers; highly trained cyclists, recreationally active) and non-exercising cohorts have not been able to replicate such findings (Crooks et al., 2010; Davison and Diment, 2010; Patiroglu and Kondolot, 2013; Shing et al., 2007, 2013). Such evidence does suggest that the lack of universal effect of COL on salivary IgA may be due to methods of IgA determination or the variance in dosage and supplementation duration with splitting the dose across the day or longer periods of supplementation (> 4 weeks) being most effective (Davison and Diment 2010; Shing et al., 2009a).

In the one study which has investigated other salivary immune parameters, it also found no benefit in resting sLys concentration following 4 weeks of COL supplementation (Davison and Diment, 2010). Given the role of mucosal immunity in conferring defence against respiratory pathogens, it may therefore be expected to see benefits to both URI and mucosal immunity (e.g. IgA) when both are measured simultaneously. To date, however, when benefits to URI incidence have been shown there has been no concurrent increase in resting salivary IgA or vice versa (Crooks et al., 2006; 2010; Patiroglu and Kondolot; Shing et al., 2007). This supports proposals that the effect of COL may occur through other or a combination of mechanisms (Shing et al., 2007).

One other reported indirect mechanism of COL on the immune system yet to be discussed is the ability of COL to maintain intestinal integrity as found in a recent PLA controlled, counterbalanced, crossover investigation (Marchbank et al., 2011). Following a strenuous exercise protocol which triggered a rise in core temperature of 1.5 - 2°C, intestinal permeability within the PLA group increased 2.5 fold whereas this rise was truncated 80% by prior COL supplementation (2 weeks of 20 $g \cdot day^{-1}$). An *in vitro* investigation ran in conjunction suggested that the *in vivo* effects may be attributable to growth factors present in COL (e.g. epidermal growth factor) which mediate apoptosis and induction of proteins (e.g. heat shock proteins) that provide cellular protection against environmental stressors. Therefore, COL may also act by preventing additional stressors on the immune system that are induced by an increase in intestinal permeability such as the translocation of luminal bacteria into the systemic circulation following strenuous exercise. Based on the above evidence it may be suggested that the benefits and mechanisms of COL as a nutritional aid to enhance human immune function and resist infection may be more apparent in the recovery from an acute stressor (e.g. exercise) that induces perturbations in both cell-mediated and mucosal immunity rather than effects on resting immune function. This may have important implications towards the hypothesis that acute windows of immunodepression rather than resting immunity provide the overall greater risk of URI in athletes.

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Table 1.2 Summary of studies that have examined the effects of COL or bovine milk derived components on PMN cells *in vitro.*

Reference	Origin of PMN	COL/milk preparation	Exposure to COL/milk	Stimulants tested	Findings
Sugiasawa et al., 2001	Bovine	0.1% - 50% COL concentrations	30 min	Fluroesbrite Carboxylate Microspheres (latex beads)	Dose dependent ↑ in phagocytosis with COL which peaked at 25% concentration and conditions of 37°C. Phagocytic activity with 25% COL greater than both milk and serum ($p < 0.01$). Phagocytosis promoting effect decreased gradually in COL obtained from 0 h to 12, 24 and 48 post-parturition.
Sugisawa et al., 2002	Bovine	0.1% - 50% COL concentrations	1-10 min	*Staphylococcus aureus* PMA	↑ in oxidative burst to *Staphylococcus aureus* with 0.1% COL concentration ($p < 0.05$). ↑ in oxidative burst with COL was greater with *Staphylococcus aureus* than PMA stimulation in comparison to control. Effect of COL on oxidative burst not dependent on pre-incubation time. Enhancement of oxidative burst not triggered by opsonisation with COL.
Sugisawa et al., 2003	Bovine	Range of molecular weight fractions of10 fold diluted COL	30 min for all fractions (findings displayed for 10 min)	*Staphylococcus aureus* PMA	Only low molecular weight fraction of COL (<10 KDa) enhanced oxidative burst ($p < 0.05$). Low molecular weight fraction primed response to *Staphylococcus aureus* ($p < 0.05$) but not PMA. Priming of oxidative burst lost in heat exposure (≥ 60°C).
Rusu et al., 2009	Human	Whey protein extract	24 h for all measures	Zymosan A fMLP	Compared to control medium dose dependent ↑ (compared to untreated PMN cells) with COL in the chemotactic, phagocytic, oxidative burst and degranulaton response to fMLP ($p < 0.05$). ↑ translocation of NADPH-oxidase subunits to plasma membrane of neutrophils with WPE.
Benson et al., 2012	Human	Low molecular weight fraction of COL (0.001-1 $g \cdot L^{-1}$)	2 min for phagocytosis 18 h for chemotaxis	Carboxylated fluroospheres fMLP	↑ in PMN cells engaged in phagocytosis ($p < 0.001$) and phagocytic activity ($p < 0.05$) of these cells compared to cells untreated and pre-exposed to fMLP and LPS. Dose dependent ↑ (compared to untreated PMN cells) with COL in migration towards fMLP ($p < 0.05$).
Jensen et al., 2012	Human	Low molecular weight fraction of COL (150)	Blood drawn 1 h and 2 h following intake of COL and PLA (white rice flour)	Not specified	↑ in phagocytic activity from baseline to 2 h post COL consumption, trend at 1h ($p = 0.08$), significant at 2 h ($p < 0.05$).

Table 1.3 Summary of studies that have examined the effect of COL on salivary IgA

Reference	Design	Population	Supplementation	Saliva collection method	IgA determination	Findings
Mero et al., 1997	DB, PC, R, C	9 male sprinters and jumpers	8 days of 125 ml COL or 25 ml COL (with 100 ml milk whey [PLA]) or 125 ml milk whey	Not specified	ELISA	No change in mean saliva IgA over 8 day period in either COL group.
Mero et al., 2002	DB, PC, R	19 active adult athletes (10 males and 9 females)	2 weeks of 20 $g.day^{-1}$ (split as four 5 g dose) of COL or maltodextrin (PLA) -participant numbers not specified	Not specified	Immunoprecipitation	↑ in mean saliva IgA concentration at end of supplementation period with COL ($p < 0.05$) but not in PLA group.
Crooks et al., 2006	DB, PC, R	35 distance runners (15 females, 20 males) aged 35-58 years	12 weeks of 12 $g.day^{-1}$ COL (n =18) compared to isomacronutrient skimmed milk (n =17)	Unstimulated passive drool	ELISA for human IgA ($IgA_1$and IgA_2)	↑ in median saliva IgA concentration at end of supplementation period with COL ($p < 0.05$) but not in PLA group. No effect of COL on IgA:osmolality.
Shing et al., 2007	DB, PC, R	29 highly trained cyclists	8 weeks of 10 $g.day^{-1}$ of concentrated COL (n = 14) or whey protein (n = 15)	Unstimulated passive drool	Nephelometry for human IgA	No change in mean IgA concentration, secretion rate or relative to albumin.
Crooks et al., 2010	DB, PC, R	25 (12 male, 13 female) elite swimmers, 28 aged-matched non-exercising CON	10 weeks of 20 $g.day^{-1}$ of low protein COL (12 swimmers, 16 CON) or isomacronutrient skimmed milk (13 swimmers, 12 CON)[1]	Unstimulated passive drool	Immunoturbidemtry	No effect of COL on saliva IgA concentration or relative to osmolality.
Davison and Diment 2010	DB, PC, R	20 recreationally active males (10 COL, 10 PLA)	4 weeks of 20 $g.day^{-1}$ of COL or isomacronutrient/isocaloric milk PLA	Unstimulated passive drool	ELISA for human SIgA	No increase in mean IgA concentration, secretion rate or relative to saliva osmolality.
Appukutty et al., 2010 Abstract only	PC, R	Active young adolescents (20 COL, 20 PLA)	6 weeks of 20 $g.day^{-1}$ of COL or skimmed milk PLA	Not specified	Not specified	↑ in saliva IgA concentration at end of 6 weeks with COL but not in PLA group.
Patiroglu and Kondolot 2013	DB, PC, R	31 (18 male, 13 female) IgA-deficient children aged 5-17 years with acute respiratory symptoms	1 week 3 sucking tablets daily of COL(14 mg and 2.2mg lysozyme) (n =16) or matched PLA (n =15) tablet (not specified)	Unstimulated passive drool	ELISA for human SIgA	Median SIgA concentration was greater (non-significant) in COL compared to PLA after the first tablet but no differences between group sat end of 7 days.
Shing et al., 2013	DB, PC, R P	16 highly trained cyclists	8 weeks of 10 $g.day^{-1}$ of concentrated COL (n = 8) or whey protein (n = 8)	Unstimulated passive drool	ELISA for human SIgA	No change in mean SIgA concentration or secretion rate.

C – crossover; DB – double blind; P - pilot study; PC – placebo controlled; R- randomised

1.4.4 Bovine colostrum and immune response to exercise

Investigation into the effect of COL on the immune response to acute, endurance exercise in humans remains unclear. Although the combined data from animal and *in vitro* stress models suggests possible immune modulatory effects in humans (Benson et al., 2012), evidence to support such findings in an exercise-induced immune dysfunction is lacking. The need for such evidence is highlighted by the many nutritional supplements which exert substantial effects within *in vitro* culture and animal studies but fail to show such beneficial effects within human athletes under double-blind PLA-controlled procedures (Walsh et al., 2011a).

COL supplementation (10 $g \cdot day^{-1}$) was found to attenuate decreases of cytotoxic/suppressor T cells and serum IgG2 during and at the end of a high intensity training period involving cumulative acute exercise bouts (Shing et al., 2007). These effects occurred following 8 weeks of supplementation with no such influence of COL on these parameters after any other acute bout within the study. Of interest, was the limited effect of COL on all other immune parameters following exercise (NKCA, salivary IgA, neutrophil surface markers, serum cytokines, serum Ig). It is difficult to separate whether the effects of COL were most effective at the 8 week stage because of the longer term supplementation or because there was an overall larger scope for intervention in and around the high-intensity period (i.e. greater immunodepression). It was apparent in this study that the effects of COL on phagocytic cells in an exercise setting was still unclear given that the value of receptor expression as a marker of human immune modulation is questionable (Albers et al., 2005).

In a study of a acute, prolonged exercise (2 h cycling at ~64% $\dot{V}O_2$ max), Davison and Diment (2010) found that 4 weeks of COL supplementation (20 g·day^{-1}) enhanced the recovery of neutrophil degranulation (stimulated elastase release at 1 h post exercise) and reduced the exercise-induced decrements in sLys concentration and secretion rate (immediately post exercise). There was no effect of COL on another aspect of mucosal immunity, salivary SIgA, but this could well be due to the lack of significant exercise-induced change (i.e. no specific pattern of immune dysfunction) in the concentration and secretion of this parameter.

It was suggested that the results may provide support to the *in vitro* hypothesis that COL contains bioactive peptides which prime or enhance innate immunity (Jensen et al., 2012; Sugiswa et al., 2003). This is supported further by the lack of direct effect of COL on the stress response to the exercise such as leukocytosis and increase in stress hormones suggested to induce falls in neutrophil function (e.g. cortisol). The lack of influence on leukocyte counts and plasma cortisol was also demonstrated following a bout of moderate exercise (1.5 h cycling bout at 50% of maximum power output proceeded by a glycogen depletion trial) in a recent study of a shorter period of COL supplementation (2 × 12.5 g for 10 days) (Carol et al., 2011). There was also no reported effect of COL compared with PLA on circulating Ig, cytokines and CRP to which the investigators concluded that there was no role for COL in preventing exercise-induced immune dysfunction. However, the ‘immune variables’ investigated and conclusions drawn within this study are confounded by several limitations. As there were no decrements in immune parameters (e.g. serum Ig) in the PLA group it would be cautious to exclude any possible impact of COL. The magnitude of the stress on the immune system within this study could also be questioned by the lack of significant lymphocytopenia post-exercise (a commonly reported change following

prolonged exercise in the literature). There were also no leukocyte function measurements undertaken and within the cytokines measured, concentrations at some timepoints were undetectable and replaced with the arbitrary lowest measurable concentration. The above study should be interpreted with caution given that mechanisms most likely to underpin the effects of COL as a nutritional countermeasure were not explored (i.e. actual measures of immune function).

1.5 Summary and aims

Prolonged exercise induces transient immune perturbations (e.g. salivary SIgA, neutrophil function) that may increase the incidence of URI. The exercise-induced immune dysfunction is primarily mediated by increases in stress hormones (noradrenaline and cortisol). To date many nutritional supplements have failed to counter the effects of prolonged exercise on the immune system. Acute carbohydrate supplementation indirectly reduces immunodepressive effects by maintaining blood glucose concentration and thus limiting increases in stress hormones. However, such effects are yet to translate into a prevention of URI incidence which has detrimental effects on athletes

There is evidence accumulating that COL reduces the incidence of URI but its effect within a model of exercise-induced immune dysfunction is unclear. The findings of *in vitro* culture studies suggest that COL contains low molecular weight components which have direct effects on neutrophils by priming oxidative burst responses to subsequent activation. It was beyond the scope of this thesis to determine the effects of various COL preparations on immune function. Nevertheless, the one study thus far which has assessed the effects of COL on neutrophil function within an exercise stress model has observed beneficial effects (enhanced recovery of stimulated

degranulation). This may suggest that the above mentioned components are in an adequate concentration in whole COL or other components (e.g. not present in whole COL but become bioavailable following digestion) enhance human immune function. If such components become bioavailable following COL consumption then it would be expected to also see priming effects on *in vitro* oxidative burst and degranulation upon acute supplementation (Study 1, Chapter 3). Study 2 (Chapter 4) further investigates whether any effects on *in vitro* neutrophil function would be more apparent following 4 weeks of COL supplementation and hence greater opportunity for the absorption or accumulation of bioactive peptides.

In addition to the primary outcomes (neutrophil function) study 1 and study 2 also investigate whether acute or longer term COL supplementation would trigger beneficial effects on mucosal parameters (salivary SIgA, sLac, sLys) following prolonged exercise. Chronic supplementation of COL may reduce the incidence of URI and also lead to changes in the above cellular and mucosal parameters at rest. Study 3 (Chapter 5) was designed to investigate the effects of 12 weeks of COL supplementation on URI incidence, salivary microbiome, innate and mucosal immunity. At present, there is conflicting evidence regarding prior infection with herpesviruses and susceptibility to URI. Study 4 (Chapter 6) aimed to determine the effect of COL on frequency, severity and duration of URI episodes in active males with or without previous EBV infection. COL has been shown *in vitro* to modulate cytokine production to promote cell-mediated immunity. Therefore, Study 5 (Chapter 7) investigates whether COL would influence an *in vivo* T-cell-mediated immune response to a novel antigen following prolonged exercise.

COL in these studies was donated from Neovite, UK, as used previously (Davison and Diment, 2010; Marchbank et al., 2011). Given that the above product encompasses all the current proposed underlying mechanism(s) (priming components, intestinal integrity), it allowed for an investigation into the potential additive and/or synergistic effects of components within COL on human immune parameters and risk of URI.

Chapter 2. General Methods

2.1 Ethics approval

All studies were approved by the University Research Ethics Committee prior to the recruitment of any participants. Participants in each study provided both verbal and written consent following information on experimental procedures. All participants were non-smokers, non-allergic to dairy products and reported no symptoms of infection or taking any medication or dietary supplements 4 weeks prior to each study. If a visit to the laboratory involved exercise, participants completed a physical activity readiness questionnaire prior to commencing the exercise bout (see Appendix A).

2.2 Determination of maximal oxygen uptake (Study 1 and 2)

Gas exchange threshold (GET) and $\dot{V}O_2$max were determined via a continuous incremental test (30 W·min^{-1} ramp rate following 3 min of unloaded baseline pedalling) to volitional exhaustion on an electrically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands). The handlebars and seat of the cycle ergometer were adjusted for participant comfort. These settings as well as the fitting of the participants' own pedals (if applicable) were maintained in all subsequent visits. Participants were instructed to reach their preferred cadence (70-100 rpm) during the 3 min unloaded baseline cadence and then maintain that cadence for the entire test. Throughout the duration of the incremental test, expired gas was analysed by the use of an online breath-by-breath gas analysis system (Jaeger Oxycon Pro, Hoechberg, Germany). The test was terminated when the participant's cadence fell 10 rpm below their preferred cadence for more than 10 s as used

previously (Vanhatalo et al., 2007). Strong verbal encouragement was provided in the later stages of the test to encourage maximal participation. For each participant $\dot{V}O_2$max was determined by the highest 30 s average during the test. GET was estimated for each participant via the V-slope method (detecting the beginning of the excess CO_2 output generated from the buffering of Hydrogen Ions) (Beaver et al., 1986). The exercise intensity was set to the power output that would elicit 15% Δ (15% of the difference between power output at GET and $\dot{V}O_2$max) which was equivalent to ~ 55-60% of the participant's $\dot{V}O_2$ max. An intensity equivalent to 55-60% $\dot{V}O_2$max was chosen as it was deemed sustainable for 2.5 h in a recreationally active population and in accordance with previously published trials of nutritional interventions and similar exercise duration (Davison and Gleeson, 2005, 2006, 2007). The use of % Δ was also used to provide a stricter control on the relative intensity as GET (also lactate threshold in most circumstances) is an important contributor to physiological responses (e.g. metabolic) during exercise and the perceived demands (i.e. rating of perceived exertion, RPE) of the exercise bout (Coggan et al., 1992; Mihevic, 1981)

2.3 Familiarisation trials (Study 1 and Study 2)

A familiarisation trial took place 7 days prior to the main trials to accustom participants to the testing procedures and ensure that the correct exercise intensity had been identified from the previous incremental test. Participants performed a 2.5 h exercise bout on the electronically braked cycle ergometer at an intensity of 15% Δ (~ 55-60% $\dot{V}O_2$max). Expired gas was analysed during the 10th, 30th, 60th, 90th and 120th min of exercise. These timepoints were used to verify that the selected workrate did elicit the target intensity. Heart rate (HR) and RPE were monitored

every 15 min during the protocol using a telemetric device (Polar S610, Polar Electro Oy, Kempele, Finland) and Borg scale (Borg, 1982) respectively. These physiological responses were used throughout the familiarisation trial. A beverage in the form of diluted cordial (4 volumes of plain water to 1 volume of cordial) was provided at 2 $mL \cdot kg^{-1}$ of BM every 15 min. The main outcomes of this trial were to habituate the participants with the physiological measurements (noted above) and the physical stress expected in the subsequent main trials as well as ensuring that the participant could main the required intensity for the duration of the trial (i.e. 2.5 h).

2.4 Blood analytical methods

2.4.1 Blood collection and treatment

Participants remained seated, performing minimal movement for 10 min prior to each blood sample with the exception of immediately post-exercise samples which were drawn within a few min of exercise cessation. All blood samples were collected by venepuncture (with a 21 gauge precision needle [Becton-Dickinson, Oxford, UK]) from an antecubital vein into the appropriate vacutainers (Becton-Dickinson, Oxford, UK); Study 1 and 2: K_3EDTA and heparin pre-treated tubes; Study 3, 4 and 5: K_3EDTA pre-treated and plain tubes.

For all studies, aliquots of K_3EDTA treated whole blood were used in haematological analysis. An aliquot of K_3EDTA treated whole blood was set aside for determining *in vitro* stimulated neutrophil oxidative burst (Study 1, 2, and 3, see section 2.4.3.1). A 1 mL aliquot and further aliquots of heparin treated whole blood were used for determination of *in vitro* stimulated neutrophil degranulation (see section 2.4.3.2) and haematological analysis (Study 1 and 2). The remaining blood in K_3EDTA and

heparin treated tubes were centrifuged at 1500 *g* for 10 min at 4°C with subsequent plasma being stored at -80°C for later analysis (Study 1 and 2: glucose, lactate, unstimulated elastase) . Blood collected in the 6 mL plain vacutainers was allowed to clot at room temperature for 1 h 20 min. Following centrifugation (1300 *g* for 10 min at 4°C), serum was stored at -80°C for later analysis (see study 3 and 4).

2.4.2 Haematological analysis

Haemoglobin, total and differential leukocyte counts were measured in each of the K_3EDTA tubes using an automated haematology analyser (Pentra 60 C+ Haematology analyser, HORIBA Medical, Montpellier, France). Determination of Haematocrit from an aliquot of heparin anti-coagulated whole blood (except Study 5 – K_3EDTA) by a standard microcentrifugation (using a Hawksley microcentrifuge) was used along with the previously attained haemoglobin concentration, to calculate changes in blood and plasma volume from pre- to post-exercise (Study 1, 2 and 5) as previously described (Dill and Costill, 1974). The measurement of blood/plasma lactate and glucose concentrations from aliquots of heparinised samples (Study 1 and 2 only; Study 5 – K_3EDTA) was performed using an automated analyser (YSI 2300 Stat Plus, Yellow Springs, Ohio, USA). Based on 10 repeated measurements of the same sample, intra assay CV for haemoglobin, haematocrit, leukocytes, lactate and glucose were 0.9%, 0.8%, 1.7%, 0.4% and 1.2% respectively.

2.4.3 Measurements of neutrophil function

2.4.3.1 *In vitro* stimulated oxidative burst activity

A small volume of whole blood from the K_3EDTA treated tubes was placed in a microcentrifuge tube and stored at room temperature (no longer than 2 h) prior to

measurement of *in vitro* stimulated neutrophil oxidative burst response to PMA and/or fMLP using a commercially available chemiluminescence (CL) kit (ABEL, Knight Scientific Ltd, Plymouth, UK) in accordance with Davison and Gleeson (2005). The sample was diluted as described below before (~ 5 min) commencing the assay. The CL per well was measured by a microplate luminometer (FLUOstar OPTIMA, BMG Labtech, Aylesbury, UK). The contents of each microplate well that contained a stimulated sample were as follows: 10 µL of diluted whole blood (blood and Hank's balanced salt solution; HBSS, without calcium and magnesium, at ratio of 1:100), 90 µL assay buffer (HBSS with calcium and magnesium), 50 µL Pholasin and 20 µL adjuvant K (substance that enhances the luminescence of Pholasin during assays involving diluted whole blood).

These mixtures were gently shaken and incubated at 37°C for 30 s in the luminometer, prior to the addition of 20 µL of PMA (5 µg mL^{-1}) or 20 µL fMLP (10 µm) to provide an end total volume of 200 µL per well, a 1:1010 final blood dilution and a PMA or fMLP concentration of 0.5 µg·mL^{-1} or 1µM respectively. Although unstimulated samples in the PMA assay were also placed through similar mixing and incubation as the stimulated wells, 110 µL of HBSS was added to wells to ensure that the end volume of each well were standardised (replacing the combination of 90 µL HBSS and PMA). In all samples which were stimulated by PMA, CL was recorded in duplicate as relative light units (RLU) at 20 s intervals for a duration of 30 min and the area under the CL curve was calculated. The area under the unstimulated CL curve for each sample was subtracted from the mean area of the duplicate stimulated sample to determine the PMA-stimulated CL.

When fMLP was the stimulant used, CL was recorded every second for 300 s. The unstimulated state in the fMLP was calculated as the mean CL of the well during the initial 2 s (Study 1 and 2) or 45 s (Study 3) (prior to addition of fMLP). The area above the stable unstimulated state was used to determine fMLP-stimulated CL. As fMLP binds to a cell receptor and does not penetrate the cell and trigger maximal stimulation (unlike PMA), neutrophils still have the capacity to respond to PMA stimulation following fMLP stimulation (see Figure 2.1 for activation of oxidative burst by these 2 agonists). In study 1 and 2, following the 300 s of the fMLP assay, 20 µL of PMA (5 $\mu g \cdot mL^{-1}$) was dispensed into the wells and CL recorded for a further 30 min. The unstimulated state in this condition was calculated as the mean CL during the initial 40 s (following addition of PMA). The area above the stable unstimulated state (over 30 min) was used to determine the combined fMLP- and PMA-stimulated CL.

To account for such responses on a per cell basis, as used previously it was assumed that the CL responses were attributable largely to the neutrophils within the samples (Morozov et al., 2003). Thus, PMA-stimulated, fMLP-stimulated and fMLP/PMA-stimulated area under the CL curve were divided by the number of neutrophils present in each well to give CL in RLU (i.e. oxidative burst response) per neutrophil. Whole blood (primarily via leukocytes) may contribute to exercise-induced increases in ROS and a state of oxidative stress (Nikolaidis and Jamurtas 2009; Powers and Jackson 2008). The unstimulated 30 min CL responses from the PMA oxidative burst assay were expressed per L of whole blood to provide a measure of spontaneous CL (a snapshot of the level of ROS) in accordance with Davison (2011) and Davison et al. (2012). This would allow for an investigation of the effect of COL on the antioxidant potential of whole blood. These unstimulated samples were also

expressed relative to neutrophils as a further indicator of any effect of COL on the basal level of CL (ROS). Based on 20 duplicated samples, intra assay CV for the fMLP, PMA and fMLP/PMA mix assay were 5.8%, 5.0% and 5.1% respectively.

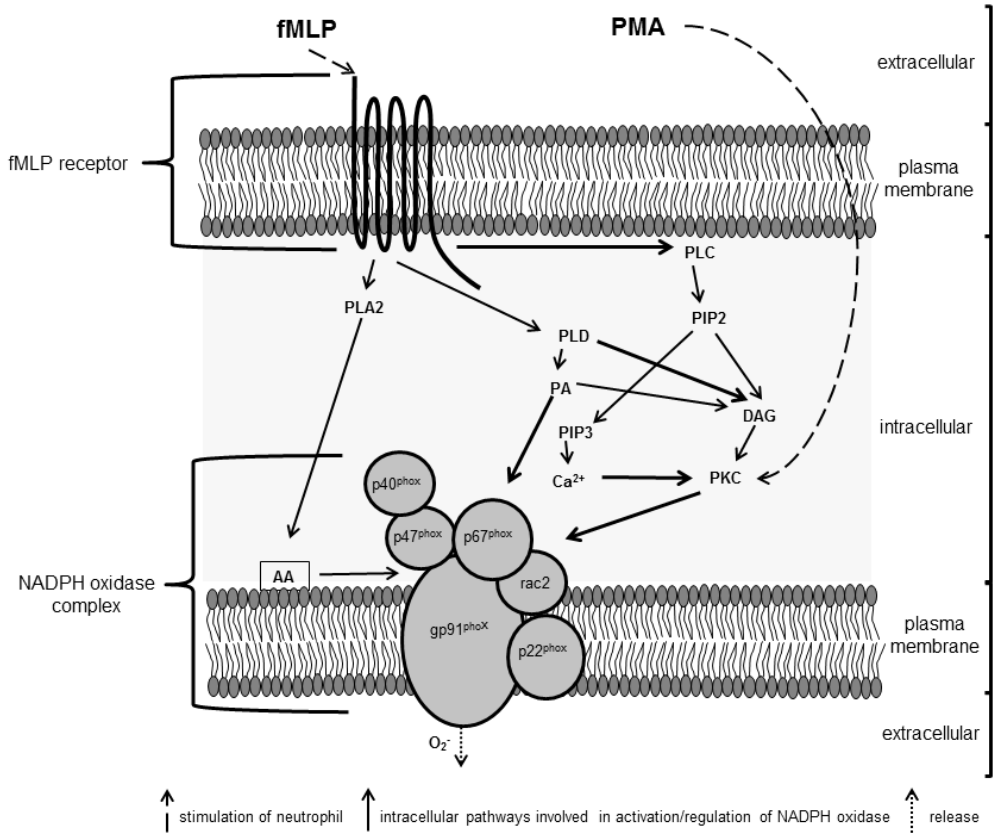

Figure 2.1. Receptor dependent (fMLP) and independent (PMA) stimulation of neutrophil oxidative burst. Attachment of fMLP to a seven transmembrane G-protein coupled receptor activates intracellular enzymes, kinases and secondary messengers (e.g. arachidonic acid, AA; calcium, Ca^{2+}; diacylglycerol, DAG; inositol phosphatidic acid, PA; phosphatidylinositol (3,4,5) triphosphate, PIP3; biphosphatphosphatidylinositol (4,5) biphosphate, PIP2; phospholipase A2, PLA2; phospholipase C, PLC; phospholipase D, PLD. These components and/or intracellular kinases (e.g. protein kinase C, PKC) aid the phosphorylation of cytosolic components (p47phox, p67phox, and p40phox; phox: phagocyte oxidase) and mediate translocation to the plasma membrane where they associate with membrane-bound components gp91phox and p22phox known as the heterodimer, llavocytochrome b558. Rac2 (signalling G protein) also migrates to the membrane to interact with p67phox and flavocytochrome b558 resulting in activation of NADPH oxidase and release of superoxide ions (O_2^-). In contrast to the rapid response of the intracellular transduction pathways to fMLP, PMA triggers a long lasting, strong stimulation via PKC which is independent of other messengers (e.g. Ca^{2+}).PMA activates NADPH oxidase on the cell surface and other parts of the neutrophil where NADPH membrane components are present (e.g. on intracellular granules). Compiled from DeCoursey and Ligeti (2005); El- Benna et al. (2005, 2008); Kanaho et al. (2013); Nick et al. (1996); Peake (2002); Selvatici et al. (2006).

2.4.3.2 *In vitro* stimulated degranulation

The neutrophil degranulation response was assessed in accordance with Davison and Gleeson (2005). The measurement of neutrophil degranulation involved adding 1 mL of the heparinised blood sample to microcentrifuge tubes containing 50 μL of bacterial stimulant (840-15, Sigma, Poole, UK). The tubes were initially mixed by gentle inversion before being incubated at 37°C for 1 h. All tubes were gently mixed halfway through the incubation period. Following incubation, the tubes were centrifuged for 2 min at 16,000 *g*, with the supernatant being immediately removed and stored at -80°C until further analysis. Upon thawing at room temperature, neutrophil degranulation response was based on measuring the amount of stimulated elastase release per neutrophil using an ELISA kit (Merck Calibiochem, Darmstadt, Germany). Bacterial-stimulated elastase release was based on subtracting elastase concentration of unstimulated samples (heparinised plasma at same timepoint) away from stimulated samples. The unstimulated samples were processed immediately to provide a 'snapshot' of the spontaneous elastase production at the specific timepoint (i.e. not incubated alongside stimulated samples). Based on 40 duplicated samples, intra assay CV was 5.3%.

2.5 Saliva collection and treatment

Participants remained seated, performing minimal movement for 10 min prior to each saliva sample with the exception of immediately post-exercise samples which were obtained within a few min of exercise cessation. For all saliva samples the mouth was rinsed with plain water at least 10 min before the collection period. The participant was requested to swallow in order to empty the mouth before each saliva sample. To obtain the sample, the participant remained seated with the head tilted

slightly forward and passively dribbling into a pre weighed 7 mL sterile bijou tube while keeping orofacial movement to a minimum. Following the 2 min, the tube was weighed again to allow for estimation of saliva flow rate when the density of saliva was assumed to be 1.0 $g.ml^{-1}$ as used in previous studies (Chicharro et al., 1998; Davison et al., 2009). When there was an insufficient amount of saliva at the end of 2 min, the process was repeated until an adequate amount was obtained. The final duration of collection was recorded to calculate saliva flow rate.

After collection of saliva, samples were centrifuged for 5 min at 16,000 *g* to pellet debris leaving the remaining clear supernatant to be aliquoted and stored at – 80°C for later analysis. All saliva samples were thawed at room temperature only once prior to analysis. Following the thawing of saliva, samples were again centrifuged for 5 min at 16,000 *g* to precipitate mucins and other debris and allow for the resulting clear supernatant to be analysed. With the use of a freezing point depression osmometer (Osmomat 030, Gonotec, GbBH, Berlin, Germany), saliva osmolality was determined to allow for concentration of salivary immunological parameters to be expressed relative to saliva osmolality. Based on 10 repeated measurements of the same sample, intra assay CV for osmolality was 1.6%.

2.6 Salivary analysis

2.6.1 Blood contamination

Aliquots of saliva were screened for blood contamination by the determination of salivary transferrin concentration using an ELISA kit (Salivary blood contamination enzyme immunoassay kit, Salimetrics, State College, Pennsylvania, USA). If salivary transferrin concentration was greater than 1 $mg{\cdot}dL^{-1}$, the sample was considered to be contaminated with blood and all other salivary data for that sample was excluded

(except SIgA due to assay being specific to the secretory component) from the study. Based on 20 duplicated samples, intra assay CV was 7.0%.

2.6.2 Salivary secretory immunoglobulin A

The concentration of salivary SIgA ($mg \cdot L^{-1}$) was determined in accordance with the protocol of previous studies (e.g. Leicht et al., 2011). Saliva supernatants were analysed using a sandwich ELISA approach with flat-bottomed microtitration plates (Nunc-ImmunoPlate; Thermo Fisher Scientific, Roskilde, Denmark). The sandwich ELISA approach was slightly modified to include coating of plates with a capture antibody specific to the secretory component of human IgA (Mouse anti-human IgA secretory, Merck Millipore, Darmstadt, Germany) before plates were blocked with a solution of 2% bovine serum albumin in phosphate-buffered saline. Duplicates of serial diluted standard (Purified IgA from human colostrum, Sigma-Aldrich) and diluted samples (1:750) were applied to plates at a volume of 50 µL and incubated overnight at 4°C. Following washing of plates, a detection antibody, was added to plates and incubated at room temperature for 90 min. After the final washing procedure, a colouring substrate (OPD substrate, Dako, UK) and a stop solution (1M $H_2SO_{4)}$ were applied in separate steps to all wells to allow for the absorbance of samples to be measured at 490 nm on a microplate reader (BioTek ELx808, BioTek Instruments, Winooski, USA). Based on 40 duplicated samples, Intra assay CV was 2.2%.

2.6.3 Salivary lactoferrin (sLac)

The concentration of sLac was measured in saliva using a commercially available enzyme-linked immunoassay technique (Assaypro LLC, St-Louis, MO). Diluted saliva samples (1:1000 with phosphate buffered saline) and serially diluted human

lactoferrin standard were assayed in duplicate. Absorbance reading was performed for all wells at 450 nm using a microplate luminometer (FLUOstar OPTIMA, BMG Labtech, Aylesbury, UK). Based on 40 duplicated samples, intra-assay CV was 2.2%.

2.6.4 Salivary lysozyme (sLys)

The concentration of sLys was measured in saliva using a commercially available enzyme-linked immunoassay technique (Assaypro LLC, St-Louis, MO). Absorbance of wells containing diluted saliva samples (1:8000 with phosphate buffered saline) and serially diluted human lysozyme standard were read on a microplate luminometer (FLUOstar OPTIMA, BMG Labtech, Aylesbury, UK). Based on 20 duplicate samples, intra assay CV was 6.5%

2.7 Monitoring of training volume

On a weekly basis within the cross-sectional design of study 3 and 4 (Chapter 5 and 6), participants were asked to complete a standard short-form International Physical Activity Questionnaire (IPAQ) (http://www.ipaq.ki.se/downloads.htm) to provide quantitative data of training loads in metabolic-equivalent (MET) $h \cdot week^{-1}$ (Craig et al., 2003).

2.8 Monitoring of upper respiratory illness

On a daily basis within the cross-sectional design of study 3 and 4, participants completed a health questionnaire (Gleeson et al., 2011, 2012a, see Appendix B). This involved participants indicating if they were suffering from any of the illness symptoms listed on the questionnaire: sore throat, catarrh in the throat, runny nose, cough, repetitive sneezing, fever, persistent muscle soreness, joint aches and pains,

weakness, headache, and loss of sleep. Upon reporting of any of the above symptoms, participants were asked to provide a subjective rating of the severity of symptoms (light, moderate, severe). As used previously (Fricker et al., 2005; Gleeson et al., 2011, 2012a), these ratings of light, moderate and severe were given numerical scores of 1, 2 and 3 respectively for data analysis. At any given point during the 12 weeks, a total symptom score of ≥12 was used to indicate that an URI was present. Participants were allowed unrestricted use of medication during episodes of URI but were asked to report such use.

2.9 General statistical analysis

Data shown in the text, tables and figures throughout this thesis are presented as mean values and/± standard deviation unless stated otherwise. Statistical analysis of all data was performed via the statistical computer software package SPSS (v20.00; SPSS Inc., Chicago, IL, USA). All data were checked for normal distribution by observations of skewness and kurtosis Z scores and using the Shapiro-Wilk test. Data not normally distributed were normalised with log or square root transformation before further analysis. Initially, either a two-factor repeated measures or a two factor mixed model ANOVA (trial × time) was carried out on all physiological and immunological measures to determine if the effect of time was different between COL and PLA (trial/group). Any significant main effects identified in the ANOVA, were further analysed by post hoc 1 tailed independent, paired t-tests or non-parametric equivalents with Holm-Bonferoni correction due to the hypothesis that COL would enhance immune parameters compared to PLA. One way ANOVA on each trial was also performed when there was evidence of trial × time interaction. Statistical significance was accepted at $p < 0.05$.

Chapter 3. Study 1- Effect of acute bovine colostrum supplementation on blood neutrophil responses and mucosal immunity following prolonged exercise

Abstract

COL supplementation for periods of 4-8 weeks has been shown to reduce the magnitude of, or speed recovery from, exercise-induced immunodepression. The purpose of this study was to identify whether acute COL supplementation prior to a bout of prolonged exercise has any effect on neutrophil function and mucosal immunity. Sixteen healthy, recreationally active males (age 25 ± 6 years; BM 75.7 ± 7.5 kg; height 178 ± 6 cm; $\dot{V}O_{2\,max}$ 54.4 ± 9.3 ml·kg^{-1}·min^{-1}; mean ± SD) participated in 2 main trials in a randomised order. Participants consumed either COL or PLA 1 h prior to 2.5 h of cycling at 15% Δ (~ 55-60% VO_2 max) (30 g), immediately prior (5 g) and midway through the exercise (5 g). Venous blood and unstimulated saliva samples were obtained prior to consumption of the supplement (BAS), 1 h post-drink (immediately pre-exercise: PRE), immediately post-exercise (POST) and 1 h post-exercise (1h POST). A greater fMLP-stimulated oxidative burst was observed in COL compared to PLA trial (trial, $p < 0.05$). There was an enhancement of sLys concentration and bacterial-stimulated neutrophil elastase release at POST and 1h POST respectively in COL trial compared with PLA trial (trial × time interaction, $p < 0.05$).There was no effect of COL on leukocyte trafficking, PMA-stimulated neutrophil oxidative burst, salivary SIgA and sLac. This study supports proposals that COL may enhance human innate immunity by a direct and immediate mechanism.

3.1 Introduction

Interest has grown in the use of COL within the area of exercise-induced immune dysfunction following findings that humans who participate in a period of supplementation (4 days – 12 weeks) benefit from enhanced prophylaxis and treatment of numerous illnesses including URI (Brinkworth and Buckley 2003; Patel and Rana, 2006; Sarker et al., 1998). Despite such findings, limited studies have investigated the effects of COL on cellular and/or innate immunity following prolonged exercise (Davison and Diment, 2010). Shing et al. (2007) when investigating leukocyte function during 8 weeks of COL supplementation, found a minor enhancement in expression of the receptor CD89 (binds IgA complexes to initiate phagocytosis and release of soluble mediators) on the surface of neutrophils during rest and following an exercise bout at end of the supplementation period. However, the value of such markers as a representation of a change in the functional capacity of leukocytes have been debated (Albers et al., 2005). Given the lack of significant neutrophilia following the performance tests during the 8 weeks of Shing et al. (2007), it can be suggested that the physical stress provided little scope to view more than modest effects of COL on neutrophil function. Previous work from our laboratory suggests that 4 weeks of COL supplementation can limit the immunodepressive effects of an acute prolonged physical stressor (2 h of cycling) by enhancing stimulated neutrophil degranulation post-exercise (Davison and Diment, 2010).

Although there is evidence to suggest that COL has an effect on the human immune system, the mechanism behind these modulations remains elusive. Recently, a shorter period of COL supplementation (10 days) was shown to have no effect on

leukocyte counts and plasma cortisol or cytokine concentrations (IL-6, IL-10, IL-1-receptor agonist, and CRP) following a 1.5 h cycling trial which was performed after a glycogen depleting bout (Carol et al., 2011). The lack of effect of COL on leukocyte trafficking and cortisol concentration supports earlier data from our laboratory (Davison and Diment, 2010), however, as Carol et al. (2011) did not measure leukocyte function, the value of shorter periods of COL supplementation remain unclear.

The findings of both animal studies and *in vitro* culture studies indicate that antimicrobial activities of neutrophils such as phagocytosis and oxidative burst are enhanced by COL (Benson et al., 2012; Sugisawa et al., 2001, 2002, 2003). The direct enhancement of functions by short term incubation with COL (~ 30 min) suggests that in the presence of COL, neutrophils are exposed to priming agents which can amplify their response upon subsequent activation. Sugisawa et al. (2003) proposed that the priming is mediated by low-molecular weight substances (< 10 kDa) present in COL. This was supported by a recent investigation by Jensen et al. (2012) where the phagocytic activity of PMN cells from healthy humans was rapidly increased 1 h following a single oral dose of a low-molecular weight fraction of COL.

In addition to the role of the neutrophils in the human body's first line of defence, salivary SIgA is an immune parameter which has been related to risk of URI in exercising populations on numerous occasions (Gleeson and Walsh 2012). COL supplementation for 6-12 weeks has been shown to increase resting levels of salivary SIgA (Appukutty et al., 2010; Crooks et al., 2006). Previous evidence from our laboratory observed no benefits of 4 weeks supplementation at rest or following prolonged exercise (Davison and Diment, 2010). This may indicate that longer term

supplementation is required to observe beneficial effects on salivary SIgA. However, Mero et al. (2002) found a 33% increase in resting salivary IgA concentration following only 2 weeks of supplementation. The daily dose of COL in this study was split across the day which may suggest an alternative mechanism towards increasing resting salivary SIgA levels. The effects of shorter but yet higher doses of COL on salivary SIgA remain unclear.

Although the importance of other salivary immune parameters (AMPs, e.g. sLys, sLac) for host defence has been recognised, they have received limited attention (West, 2006). The only study to date which has looked at the effects of COL on AMPs found a 4 week supplementation period prevented an exercise-induced decrease of sLys (Davison and Diment, 2010). This coincided with an improved recovery of neutrophil degranulation. Given the role of neutrophils as a source of AMPs (Travis et al., 2001), any acute, direct effects on neutrophil capacity may also lead to improvements in innate mucosal parameters (Davison et al., 2009).

It is currently unknown whether the above mentioned priming and 'immune-enhancing' ability will present with acute oral supplementation of COL in an exercise setting. Hence, the primary aim of this study was to determine the influence of acute oral COL supplementation on neutrophil function (degranulation and oxidative burst) following prolonged exercise. Secondly, this study also aimed to determine if COL had any effects on parameters of mucosal immunity.

3.2 Methods

3.2.1 Pilot study

Pilot work was carried out to determine a duration and dosage of COL that could potentially act as an acute intervention in human volunteers under conditions of exercise-induced immune dysfunction. Following an overnight fast, 3 males reported to the laboratory on 2 occasions separated by 7 days. On each occasion, participants either consumed 30 g of COL or 30 g of PLA (isoenergetic mixture of milk protein concentrate and skimmed milk powder) mixed in 300 mL water in a randomised double-blind manner. Solutions were also flavoured with vanilla flavouring (MP flavouring, My Protein, Northwich, UK) and were indistinguishable in flavour or appearance. Resting blood samples were taken from an antecubital vein immediately prior (PRE) to consumption of COL or PLA as well as 1 h and 3 h post-consumption. Participants remained fasted until after the 3 h blood sample was taken. At each timepoint, PMA-stimulated oxidative burst and neutrophil count was assessed in whole blood collected from the K_3EDTA vacutainer. This preliminary work suggested that COL may have a direct, acute effect on the functional capacity of blood neutrophils (Table 3.1). Compared to PLA, a greater increase in stimulated oxidative burst with COL was evident at 1 h post consumption which was maintained for a further 2 h later. A larger scale study was then completed to determine whether these benefits on neutrophil function are observed following acute COL supplementation under conditions of exercise-induced perturbations in innate immunity.

Table 3.1 Pilot study to assess acute effects of COL on neutrophil function at rest.

Supplement	PMA-stimulated CL per neutrophil (% of PRE)		
	PRE	1 h	3 h
COL	100 ± 0	133 ± 31	134 ± 29
PLA	100 ± 0	92 ± 32	110 ± 38

3.2.2 Participants

Sixteen healthy, recreationally active males (age 25 ± 6 years; height 178 ± 6 cm; BM 75.7 ± 7.5 kg; $\dot{V}O_{2\,max}$ 54.4 ± 9.3 ml·kg^{-1}· min^{-1}) volunteered to participate in the present investigation.

3.2.3 Preliminary visits

A subset of participants (n = 7) were asked to attend the laboratory for 2 visits separated by 7 days. On each visit participants consumed either 30 g of COL or PLA (solutions prepared as above). K_3EDTA anti-coagulated blood obtained 1 h following consumption of these solutions was immediately centrifuged at 1500 *g* for 10 min at 4°C with subsequent plasma stored at -80°C for later use within experimental trials (plasma incubation experiments, see section below, 3.2.4).

3.2.4 Experimental trials

At least 7 days following the preliminary visits (see previous section and sections 2.3) participants performed 2 trials separated by 7 days, in a double-blind randomised counterbalanced design. During the 48 h preceding each experimental

trial, participants were asked to refrain from heavy exercise and alcohol consumption while recording their food intake in food diaries in an effort to standardise their nutritional status in the 24 h before the first experimental trial. Participants were required to follow the same food intake during the 24 h prior to the second experimental trial. On the day of the main trial, they also completed a food frequency questionnaire relating to the previous 7 days. The dietary records were analysed using computer dietary analysis software (CompEat, Nutrition Systems, London, UK).

On the morning of each trial, participants reported to the laboratory after an overnight fast of at least 10 h. The participants were asked to consume 500 mL of water 2 h before arrival to ensure euhydration. Upon arrival, participants were asked to remain seated for 10 min prior to collection of a resting baseline blood sample (BAS) from an antecubital vein and an unstimulated saliva sample. For one trial, participants then consumed 30 g of COL (prepared as above) while the other trial involved the consumption of 30 g of PLA (prepared as above). Participants undertook restful activities (i.e. reading) in the laboratory before collection of a venous blood sample and unstimulated saliva sample (PRE) 1 h post consumption of COL or PLA. Immediately prior to the exercise participants consumed a further 5 g of COL or PLA mixed in 50 mL of water and vanilla flavouring (see above).

In each trial participants performed a 2.5 h of cycling at 15% Δ. All participants also received a further 5 g of COL or PLA at 1 h 15 min into the exercise of the respective trial. With the exception of this time point, participants were permitted diluted cordial (four volumes of water to 1 volume of sugar-free cordial at 2 $mL \cdot kg^{-1}$ of BM) during the first trial with the pattern of intake and total volume consumed being replicated in

the second trial. Expired gas was analysed during the 30th, 60th, 90th and 120th min of exercise (Jaeger Oxycon Pro, Hoechberg, Germany). HR and RPE were monitored every 15 min during the protocol using a telemetric device (Polar S610, Polar Electro Oy, Kempele, Finland) and Borg rating scale (Borg, 1982) respectively. The mean of each of the physiological responses was used to determine if similar physical stress was imposed in each trial. A venous blood sample and an unstimulated saliva sample were collected immediately post-exercise (POST). Participants remained fasted until a further blood and saliva sample at 1 h post-exercise (1h POST). To explore a proposed direct enhancement of neutrophil oxidative burst by components (Sugisawa et al., 2003) or metabolites of COL which become bioavailable following consumption, plasma obtained from the preliminary visits (section 3.2.3, i.e. 1 h following COL and PLA consumption) was thawed and incubated with whole blood (1:1 ratio, 100 μl each) (same participants as section 3.2.3) at BAS and POST in the PLA trial only. Following an incubation period of 30 min at 37°C, blood was immediately analysed for fMLP oxidative burst as detailed in 2.4.3.1.

3.3 Results

3.3.1 Nutrient intake

In the 7 days prior to the 2 experimental trials, there was no significant difference in total energy ($p = 0.443$) and macronutrient intake of the participants (carbohydrate: $p = 0.318$; fat: 0.554; protein: $p = 0.891$). The mean daily macronutrient intake (as a percentage of total energy intake) prior the COL trial was carbohydrate 52.1 ± 5.9%, fat 32.3 ± 7.0% and protein 15.6 ± 3.9%. Prior to the PLA trial the mean daily

macronutrient intake was carbohydrate 50.9 ± 7.2%, fat 32.9 ± 6.3 % and protein 15.8 ± 3.3%.

3.3.2 Physiological variables

There was no significant difference in oxygen uptake ($\dot{V}O_2$) (p = 0.218; p = 0.138) between the COL (2336 ± 279 mL·min^{-1}; 57.0 ± 4.2% $\dot{V}O_2$max) and PLA trial (2297.8 ± 274.6 mL·min^{-1}; 55.8 ± 4.5% $\dot{V}O_2$max). The average HR (134 ± 15 bpm and 132 ± 14 bpm during COL and PLA trials respectively) were not significantly different (p = 0.209). There was no significant difference in average RPE (p = 0.174) between the COL (12.9 ± 1.3) and PLA (12.6 ± 0.8) trials. A similar pattern of plasma volume changes was observed from BAS between trials: COL; PRE (0.5 ± 4.3%); POST (-3.7 ± 5.6%); 1h POST (-0.1 ± 4.5%) and PLA; PRE (-1.7± 2.1%); POST (-5.4 ± 3.1%), 1h POST (-2.0 ± 5.6%). As there was no significant difference between trials (trial × time interaction; p = 0.915), it was deemed unnecessary to correct any haematological parameters for plasma volume changes.

Although there was a significant change across time (p < 0.001), there was no significant trial (p = 0.073) or interaction effect (p = 0.694) on plasma glucose (Table 3.2). Plasma glucose was significantly lower at PRE (p < 0.001), POST (p = 0.003) and 1h POST (p < 0.001) compared to BAS. There was also a significant change across time for plasma lactate (p < 0.001) but no effect of trial (p = 0.970) or interaction (p = 0.381) (Table 3.2). Plasma lactate significantly increased from BAS (p = 0.001) and PRE (p < 0.001) to POST before decreasing towards resting levels at 1h POST (p = 1.000).

Table 3.2 Plasma glucose and lactate responses following acute COL or PLA consumption.

Measure, mmol·L^{-1}	BAS	PRE	POST	1h POST	p values trial time interaction
Plasma glucose					0.073
COL	5.10 ± 0.39	4.39 ± 0.57	4.38 ± 0.42	4.25 ± 0.39	< 0.001*
PLA	5.19 ± 0.51	4.43 ± 0.55	4.56 ± 0.53	4.35 ± 0.34	0.694
Plasma lactate					0.970
COL	1.50 ± 0.48	1.37 ± 0.29	2.04 ± 0.71	1.69 ± 0.66	< 0.001*
PLA	1.49 ± 0.38	1.42 ± 0.26	1.97 ± 0.69	1.60 ± 0.43	0.381

*Significant main effect of time ($p < 0.001$).

3.3.3 Immune cell counts

No significant trial or interaction effects were evident for total or differential leukocyte counts (Table 3.3). A main effect of time was observed in all leukocyte counts (Table 3.3). There was a significant increase in total leukocytes, neutrophils, monocytes, neutrophil:lymphocyte ratio and large immature cells from BAS to POST and 1h POST ($p \leq 0.001$). Total lymphocyte count at PRE was significantly lower than BAS ($p < 0.01$) and POST ($p < 0.001$). There was a significant increase in neutrophil:lymphocyte ratio ($p < 0.001$) and decrease in total lymphocytes and monocytes ($p < 0.01$) from POST to 1h POST ($p < 0.01$).

Table 3.3 Immune cell counts following acute COL or PLA consumption.

Cell count, $10^9 \cdot L^{-1}$	BAS	PRE	POST	1h POST	p values trial time interaction
Total leukocytes					0.255
COL	5.38 ± 1.46	5.51 ± 1.44	12.98 ± 5.01	12.15 ± 1.09	< 0.001*
PLA	5.45 ± 1.33	5.17 ± 1.00	11.80 ± 3.43	11.43 ± 2.86	0.349
Neutrophils					0.566
COL	2.77 ± 1.15	3.23 ± 1.28	9.38 ± 4.19	9.54 ± 3.96	< 0.001*
PLA	2.80 ± 0.79	2.98 ± 0.83	8.56 ± 2.96	8.86 ± 2.49	0.495
Monocytes					0.820
COL	0.50 ± 0.15	0.47 ± 0.13	0.90 ± 0.32	0.75 ± 0.23	< 0.001*
PLA	0.53 ± 0.16	0.46 ± 0.12	0.82 ± 0.28	0.72 ± 0.19	0.211
Total lymphocytes					0.073
COL	1.86 ± 0.45	1.62 ± 0.39	2.41 ± 0.74	1.77 ± 0.27	< 0.001*
PLA	1.89 ± 0.67	1.53 ± 0.33	2.16 ± 0.62	1.63 ± 0.39	0.176
Neutrophil:lymphocyte					0.454
COL	1.55 ± 0.67	2.13 ± 1.06	3.95 ± 1.36	5.49 ± 2.13	<0.001*
PLA	1.59 ± 0.59	2.03 ± 0.78	4.27 ± 2.01	5.62 ± 1.68	0.962
Large immature cells					0.870
COL	0.04 ± 0.02	0.04 ± 0.02	0.20 ± 0.14	0.18 ± 0.13	< 0.001*
PLA	0.05 ± 0.02	0.04 ± 0.02	0.17 ± 0.10	0.15 ± 0.06	0.402

*Significant main effect of time ($p < 0.001$).

3.3.4 Neutrophil responses

A significant main effect time of time ($p < 0.001$) was observed for fMLP-stimulated CL per neutrophil (neutrophil oxidative burst) (Figure 3.1A). There was a significant decrease in fMLP-stimulated CL per neutrophil compared to BAS at PRE ($p = 0.020$), POST ($p < 0.001$) and 1h POST ($p < 0.001$). There was a main effect of trial for fMLP-stimulated CL per neutrophil ($p = 0.024$) but no trial ^{x} time interaction ($p = 0.158$ (Figure 3.1A).

A significant main effect of time ($p < 0.001$) was observed for PMA-stimulated CL per neutrophil (neutrophil oxidative burst) (Figure 3.1B). There was a significant decrease in PMA-stimulated CL per neutrophil compared to BAS at POST ($p = 0.001$) and 1h POST ($p < 0.001$). There was no main effect of trial ($p = 0.449$) or trial ^{x} time interaction ($p = 0.741$) for PMA-stimulated CL per neutrophil (Figure 3.1B). Two-way ANOVA on PMA-stimulated CL per neutrophil following fMLP stimulation showed a main effect of time ($p = 0.005$) but no effect of trial ($p = 0.335$) or interaction ($p = 0.344$) (Table 3.4). Post hoc analyses could not reveal any significant differences between timepoints, only a trend for POST to be lower than BAS ($p = 0.094$).

A significant main effect of time ($p < 0.001$) was observed for unstimulated CL per neutrophil (basal ROS) (Table 3.4). There was a significant decrease in unstimulated CL per neutrophil compared to BAS at PRE ($p = 0.008$), POST ($p < 0.001$) and 1h POST ($p = 0.001$). There was no main effect of trial ($p = 0.495$) or trial ^{x} time interaction ($p = 0.655$) for unstimulated CL per neutrophil (Table 3.4). There was no main effect of time ($p = 0.426$) trial ($p = 0.785$) or trial ^{x} time interaction ($p = 0.692$) for spontaneous $CL \cdot L^{-1}$ blood (Table 3.4).

There was no significant main effect of trial ($p = 0.257$) or time ($p = 0.135$) for stimulated elastase release per neutrophil (neutrophil degranulation) (Table 3.4). However, there was a significant difference within trials for exercise-induced changes in stimulated elastase release per neutrophil (trial ^{x} time interaction, $p = 0.01$) (Table 3.4). A one-way ANOVA on each trial showed a time effect in the PLA trial ($p = 0.009$) but not in COL trial ($p = 0.547$). Further post hoc analysis of PLA trial showed there was a significant decrease in stimulated elastase release from PRE to 1h POST ($p = 0.029$).

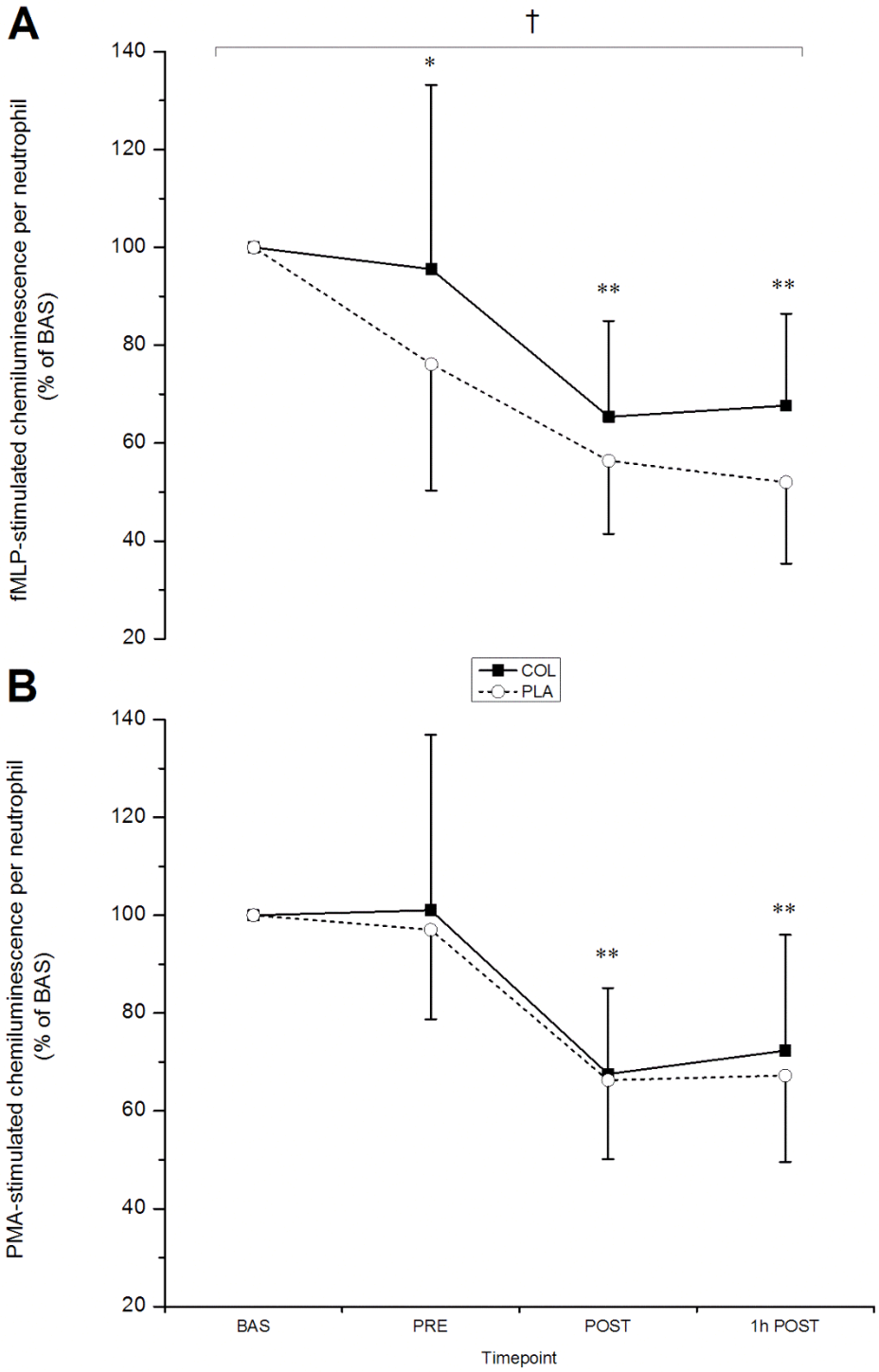

Figure 3.1 fMLP (A) and PMA (B) stimulated chemiluminescence per neutrophil following acute COL or PLA consumption. Significant change from BAS (*$p < 0.05$, **$p < 0.001$). †Significant main effect of trial for fMLP-stimulated chemiluminescence per neutrophil ($p < 0.05$).

Table 3.4 Neutrophil responses following acute COL or PLA consumption.

Measure	BAS	PRE	POST	1h POST	p values trial time interaction
PMA-stimulated CL following fMLP stimulation per neutrophil (% of BAS)					0.355
COL	100 ± 0	93.0 ± 18.4	82.1 ± 34.6	92.9 ± 36.1	< 0.001*
PLA	100 ± 0	96.3 ± 7.7	74.3 ± 33.6	73.7 ± 27.2	0.344
Unstimulated CL per neutrophil (% of BAS)					0.495
COL	100 ± 0	87.6 ± 28.5	33.8 ± 14.3	32.1 ± 13.8	< 0.001*
PLA	100 ± 0	86.2. ± 16.5	39.2 ± 19.0	35.4 ± 22.4	0.665
Spontaneous $CL \cdot L^{-1}$ blood					0.785
COL	964 ± 360	946 ± 323	1008 ± 307	960 ± 289	0.426
PLA	1020 ± 394	943 ± 449	1106 ± 516	1084 ± 731	0.692
Stimulated elastase release per neutrophil ($fg \cdot cell^{-1}$)					0.257
COL		401 ± 168	366 ± 143	396 ± 191	0.135
PLA		467 ± 179	418 ± 105	356 ± 119†	0.010

*Significant main effect of time ($p < 0.001$). †Significant main effect of interaction ($p < 0.05$).

3.3.5 Plasma incubations

Two-way ANOVA analysis revealed a significant main effect of treatment for fMLP-stimulated oxidative burst ($p = 0.026$) but no effect of time ($p = 0.087$) or treatment × time interaction ($p = 0.293$). The observed effects indicated an overall higher response to fMLP across both timepoints (BAS and POST) when whole blood (i.e. neutrophils) was pre-incubated with plasma obtained 1 h following COL consumption (Table 3.5). It is worthy to note that the main focus here was to observe the main effect of COL as the temporal pattern (i.e. interaction effect) may be confounded by enhancements with COL at both timepoints and a lack of statistical power compared to section 3.3.4 to identify an effect of time (i.e. exercise).

Table 3.5 CL (oxidative burst) responses following pre-incubation of whole blood from PLA main trial with plasma collected following COL or PLA consumption.

fMLP- stimulated CL per neutrophil ($RLU \cdot s^{-1}$ $cell^{-1}$)	COL	PLA	p values trial time interaction
			0.026*
BAS	32.8 ± 12.1	25.3 ± 13.7	0.087
POST	24.0 ± 10.7	21.0 ± 9.1	0.293

*Significant main effect of trial ($p < 0.05$)

3.4 Saliva SIgA and antimicrobial peptide responses

There was no significant main effect of trial ($p = 0.604$) or interaction ($p = 0.518$) for salivary SIgA concentration (Table 3.6). There was a significant main time effect ($p = 0.002$) with post hoc analysis showing that salivary SIgA concentration was lower at PRE compared to BAS ($p = 0.006$) and POST ($p = 0.039$). Two-way ANOVA revealed no significant main effects of time ($p = 0.315$), trial ($p = 0.384$) or interaction ($p = 0.220$) for salivary SIgA secretion rate (Table 3.6). There was no main effect of trial ($p = 0.205$) or interaction ($p = 0.150$) for saliva SIgA:osmolality (Table 3.6). There was, however, a main effect of time ($p < 0.001$) with post hoc analysis showing a significant decrease in SIgA: osmolality from BAS to POST ($p < 0.001$) while decreases from BAS to PRE ($p = 0.057$) and increases from POST to 1h POST ($p = 0.071$) approached significance.

There was no significant main effect of trial ($p = 0.602$) or interaction ($p = 0.327$) for sLac concentration (Table 3.6). There was a significant main time effect ($p = 0.001$) with post hoc analysis showing that sLac concentration was increased at POST compared to BAS ($p = 0.033$) and PRE ($p = 0.039$). ANOVA also revealed a main

effect of time for sLac secretion rate (p = 0.044) but post hoc analysis could not identify any specific differences between time points (Table 3.6). There was no effect of trial (p = 0.549) or interaction (p = 0.813) on sLac secretion rate. There was a main effect of time (p = 0.022) with sLac:osmolality but post hoc analysis could not identify specific differences between timepoints (Table 3.6). There was no main effect of trial (p = 0.855) or interaction (p = 0.067) for sLac:osmolality.

Two-way ANOVA showed a significant interaction (trial × time) for sLys concentration (p = 0.018) (Figure 3.2). Paired t-tests showed there was a higher sLys concentration at POST in the COL trial compared to PLA trial (p = 0.007). This was supported by a one-way ANOVA on each trial showing a main effect of time (0.026) with significantly greater sLys concentration at POST compared to PRE in COL trial (p = 0.031) while there was no significant change across time for sLys concentration in PLA trial (p = 0.766). There was no main effect of time (p = 0.333) but the effect of trial approached significance (p = 0.059). There was a trend (p = 0.061) for an interaction (trial ×time) for sLys secretion rate but no effect of trial (p = 0.254) or time (p = 0.528) (Table 3.6). Two-way ANOVA showed a significant interaction (trial × time) for sLys:osmolality (p = 0.042) (Figure 3.3). Paired t-tests showed there was a higher sLys:osmolality at POST in the COL trial compared to PLA trial (p = 0.013). One-way ANOVA on each trial revealed no significant main effects of time in COL (p = 0.093) or PLA trial (p = 0.312). No significant main effects of trial (0.226) or time (0.356) were reported for sLys:osmolality.

Table 3.6 Changes in salivary SIgA and AMPs with acute COL or PLA supplementation.

Immune measure	BAS	PRE	POST	1h POST	*p* value trial time interaction
SIgA concentration ($mg \cdot L^{-1}$)					0.604
COL	364 ± 177	317 ± 201	359 ± 350	350 ± 191	0.002*
PLA	385 ± 183	301 ± 151	352 ± 175	374 ± 162	0.518
SIgA secretion rate ($\mu g \cdot min^{-1}$)					0.384
COL	160 ± 95	196 ± 116	179 ± 119	191 ± 105	0.315
PLA	161 ± 106	159 ± 94	179 ± 132	194 ± 131	0.220
SIgA: osmolality ($mg \cdot mosmol^{-1}$)					0.205
COL	5.3 ± 1.8	4.8 ± 2.6	4.1 ± 1.2	4.5 ± 1.8	< 0.001**
PLA	5.4 ± 1.8	4.6 ± 1.7	4.2 ± 1.3	5.2 ± 1.6	0.150
sLac concentration ($mg \cdot L^{-1}$)					0.602
COL	3.9 ± 0.8	4.0 ± 0.9	4.9 ± 1.0	4.3 ± 1.1	0.001*
PLA	3.7 ± 1.0	4.3 ± 1.0	4.9 ± 0.9	4.5 ± 1.1	0.327
sLac secretion rate ($\mu g \cdot min^{-1}$)					0.549
COL	2.3 ± 1.5	3.2 ± 2.0	2.9 ± 1.7	3.1 ± 1.8	0.044*
PLA	2.0 ± 1.7	3.1 ± 1.9	2.8 ± 1.5	2.7 ± 1.3	0.813
sLac: osmolality ($mg \cdot mosmol^{-1}$)					0.855
COL	0.06 ± 0.01	0.06 ± 0.01	0.05 ± 0.02	0.06 ± 0.02	0.022*
PLA	0.05 ± 0.02	0.06 ± 0.01	0.06 ± 0.01	0.06 ± 0.02	0.067
sLys secretion rate ($\mu g \cdot min^{-1}$)					0.528
COL	10.0 ± 7.3	12.9 ± 7.1	17.2 ± 13.4	12.6 ± 12.0	0.254
PLA	9.8 ± 11.5	11.9 ± 8.2	8.8 ± 6.7	11.4 ± 8.2	0.061

Significant main effect of time (* $p < 0.05$, ** $p < 0.001$).

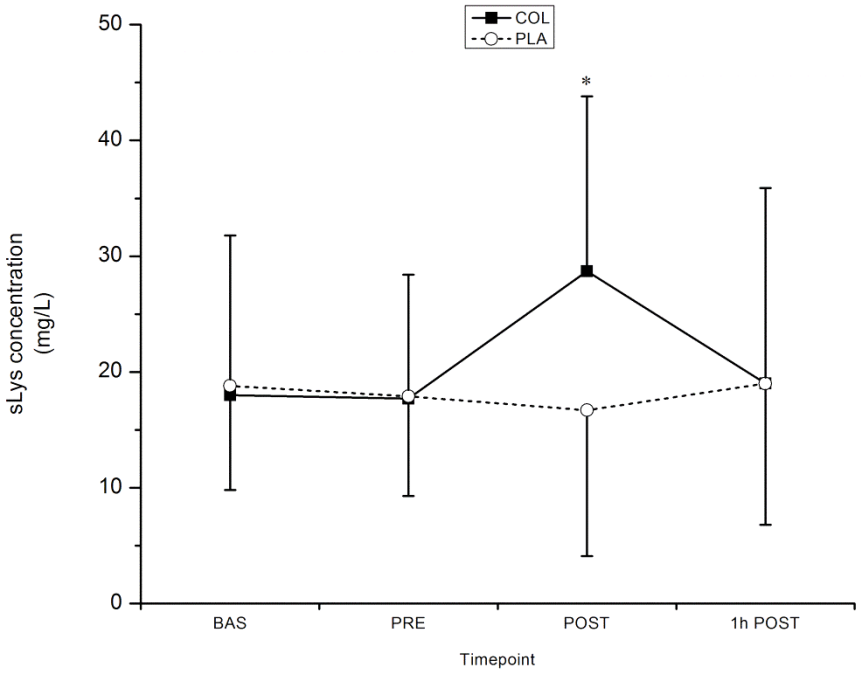

Figure 3.2 The response of sLys concentration following acute COL or PLA supplementation. *Significant difference between trials ($p < 0.05$).

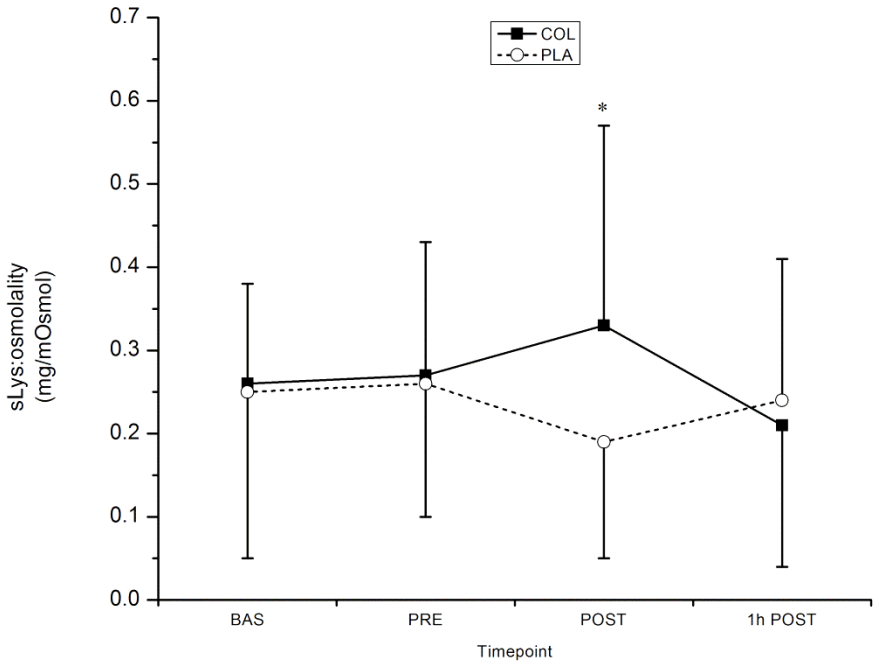

Figure 3.3 The response of sLys:osmolality following acute COL or PLA supplementation. *Significant difference between trials ($p < 0.05$).

3.5 Discussion

The main finding(s) of this study was that acute consumption of COL resulted in a greater fMLP-stimulated oxidative burst compared to PLA and an enhancement of bacterial-stimulated neutrophil degranulation and salivary lysozyme in the recovery period following prolonged exercise. This supports previously mentioned benefits of COL on the human innate immune system following prolonged exercise (Davison and Diment, 2010) but it is the first study to demonstrate such effects with acute COL supplementation.

Only one earlier study had investigated a brief period (10 days) of COL supplementation on leukocyte responses to prolonged exercise. Carol et al. (2011) suggested no role of COL supplementation in preventing exercise-induced immune dysfunction despite lacking any measurements of leukocyte function. Similar to previous research a significant immunological stress was observed in response to prolonged exercise during the COL and PLA trials with increases in leukocytes, neutrophils, lymphocytes (followed by lymphocytopenia), neutrophil:lymphocyte ratio, monocytes and large immature cells (Davison and Diment, 2010; Lancaster et al., 2003; Li and Gleeson, 2004, 2005; McCarthy and Dale, 1988; Nieman, 1998).

The evidence of a greater fMLP-stimulated oxidative burst in the COL trial and greater recovery of neutrophil degranulation at 1 h post-exercise compared to PLA suggest that acute COL supplementation may have similar benefits to chronic supplementation (Davison and Diment, 2010). Similar to previous studies on COL supplementation there was no apparent effect of COL on exercise-induced leukocytosis (Carol et al., 2011); supporting proposals from *in vitro* evidence that COL may have direct effects on leukocyte function (Davison and Diment, 2010;

Sugisawa et al., 2003). The fact that PMA-stimulated oxidative burst was unaffected by COL may provide a novel insight into how COL modulates neutrophil activity following exercise-induced immune dysfunction. Evidence from numerous *in vitro* studies suggest that short pre-term incubation with COL primes the response of neutrophils to subsequent stimulation (Benson et al., 2012; Sugisawa et al., 2001, 2002, 2003). Neutrophil priming has been demonstrated with a range of certain cytokines (GM-CSF, G-CSF, IL-8, TNFα, (Elbim et al., 1994; You et al., 1991; Khwaja et al., 1992). The exact intracellular events that occur with these priming agents depend on the subsequent activating agonist of neutrophil stimulation but it seems that priming involves phosphorylation of cytosolic proteins that are crucial in the assembly of NADPH oxidase on the plasma membrane (Dang et al., 1999; Dewas et al., 2003). In line with changes of *in vitro* neutrophil activation observed in this study, some of these pro-inflammatory cytokines (e.g. TNFα) can prime the receptor-mediated stimulation (degranulation and oxidative burst) of neutrophils by bacterial products but not affect receptor-independent responses to synthetic stimuli (e.g. PMA) (Bajaj et al., 1992).

Despite the reported effects *in vitro*, the priming agent within COL remains unclear. Sugisawa et al. (2003) proposed that priming effects are due to low molecular weight components such as proteose peptones following findings that a COL fraction containing substances less than 10 kDa enhanced responses to *Staphylococcus aureus* whereas fractions containing constituent cytokines (IL-1, IL-6, TNFα) of COL did not have any effects. Of note to the present study was that the priming nature of this low molecular weight fraction in Sugisawa et al. (2003) was lost when neutrophils were stimulated with PMA. Benson et al. (2012) observed enhancement of *ex vivo* neutrophil phagocytosis 1 h and 2 h following consumption of a low

molecular weight fraction of COL in healthy males. As any priming components remain unclear, the direct or indirect effects of COL on cytokines *in vivo* cannot be excluded due to the molecular mass of some cytokines with mediating effects on neutrophils being < 10 kDa (e.g. IL-8) (Lin et al., 2004; Mikami et al., 1998). Furthermore, COL is considered to be a precursor of numerous potential biologically active peptides (Jorgensen et al., 2010). Although IgG within non-digested COL is unlikely to pass into circulation, the digestion of its Fc heavy chain may release other metabolites (e.g. Tuftsin) which have been shown to modulate neutrophil oxidative burst (Najjar and Nishioka, 1980; Siemion and Kluczyk, 1999; Werner et al., 1986; Wu et al., 2012). Such mechanism(s) triggered by metabolites of COL would challenge hypotheses proposed by others (Sugisawa et al., 2001, 2002, 2003) that the component is present within whole COL. Nevertheless, this study expands on previous *in vitro* co-culture evidence by suggesting that these 'priming' and/or immune- enhancing components become biologically available to affect neutrophil function upon oral consumption of COL within a model of exercise-induced immune dysfunction. These findings are further supported by the enhancement of neutrophil oxidative burst when neutrophils are pre-incubated with plasma collected following acute COL consumption. It can be suggested from the findings presented here that the mechanism(s) of acute COL supplementation are not due to a modulation of antioxidant capacity or basal ROS production due to a lack of effect on unstimulated or spontaneous oxidative burst. Based on the available evidence, it can be speculated that the enhancement of neutrophil function by COL is mediated by changes proximal to surface receptors and/or intracellular signal transduction pathways that are independent of PKC as responses to PMA-stimulated (strong PKC agonist, Sheppard et al., 2005) were unaffected.

This hypothesis, however, would not explain the observed enhancement of PMA-stimulated oxidative burst 1-3 h following COL consumption in resting males in the pilot study. However, it must be recognised that previous *in vitro* work by others has suggested that priming effects of COL on PMA-stimulated oxidative burst responses are absent (Sugisawa et al., 2003) or mild and substantially lower (Sugisawa et al., 2002) compared to responses to bacterial (*Staphylococcus aureus*) stimulation. Physiological stimuli like fMLP can stimulate neutrophils multiple times while PMA is considered a maximal stimulus which migrates into the cell and stimulates ROS production via all intracellular sources (Sheppard et al., 2005). In the experimental trial of this study, there was no beneficial effect of COL on PMA-stimulated oxidative burst 1 h post consumption of COL or in post exercise measures. There was also no effect of COL on PMA-stimulation when neutrophils had previously been activated by fMLP. However, as measurement of response to PMA following fMLP stimulation was only carried out on a small subset of participants (n = 8) and potentially limited by a lack of statistical power, further investigation is warranted. Taken together, it can be suggested that components of COL and/or its metabolites may possess the ability to prime neutrophil responses by mediating various pathways of signal transduction but the functional significance of these may be influenced by exercise.

The importance of mucosal immune parameters such as AMPs towards host defence have been recognised (West et al., 2006). In accordance with Davison and Diment (2010), COL compared with PLA enhanced sLys concentration immediately-post exercise, before the parameter returned to pre-exercise levels in both trials after 1 h of recovery. The effects occurred despite the lack of exercise-induced decrease in sLys in the present study that was observed in Davison and Diment (2010). Such findings may provide further evidence to the aforementioned proposal regarding COL

and the effector responses of neutrophils. In addition to circulating netrophilia, it has been suggested that there is an increase in the number of neutrophils in mucosal secretions following prolonged exercise (Muns, 1994). As neutrophils are considered an important source of sLys (Travis et al., 2001), it is plausible that the increased concentrations post-exercise are due to the migration of 'primed' neutrophils into the oral cavity. sLys along with sLac are the most abundant AMPs within saliva (Singh et al., 2000). In contrast to sLys, there was increased sLac concentration post-exercise in both the COL and PLA trials. These exercise-induced changes, however, are in agreement with other investigations of sLac or other AMPs that have similar sources (e.g. LL37) (submucosal glands and epithelial cells) (Davison et al., 2009; Gillum et al., 2013). The functional significance of these increases is unclear at present but it may reflect an inflammatory response to exercise-induced damage to mucosal surfaces. Given the suggested synergistic effects of these AMPs on host defence within the oral cavity, further research is required to elucidate any influence by COL.

There was also no apparent effect of COL on salivary SIgA in this study. Within this study design, benefits to salivary SIgA may have been via a modulation of responses to exercise or an elevation in pre-exercise levels. Similar to a 4 week supplementation period in Davison and Diment (2010), none of these effects were seen with acute COL intake. Based on evidence from studies that have observed increases in resting salivary SIgA with COL (Crooks et al., 2006; Mero et al., 2002), longer term supplementation (e.g. 12 weeks) and/or splitting the dose across the day may be required. In accordance with others (Allgrove et al., 2009; Davison et al., 2009), there was a decrease in saliva SIgA:osmolality post-exercise which highlights the potential perturbations on mucosal immunity following prolonged exertion. However, the lack of simultaneous decreases in salivary SIgA concentration or

secretion rate reiterates the variance and/or discrepancies with salivary SIgA expression methods (Bishop and Gleeson, 2009).

A possible limitation to the present study was that there was a lack of or minimal exercise-induced immune dysfunction in some parameters (e.g. salivary SIgA) providing little scope for benefit via a nutritional countermeasure (i.e. COL). Despite significant falls with *in vitro* fMLP- and PMA-stimulated neutrophil oxidative burst supporting previous findings (e.g. Davison and Gleeson, 2005; Henson et al., 2008), the lack of simultaneous decrease in bacterial-stimulated degranulation immediately post-exercise is somewhat surprising and dissimilar to many previous findings (e.g. Davison and Gleeson, 2005, 2006; Laing et al., 2005, 2008). It is likely that the cumulative intensity and duration of the current exercise protocol was not strenuous enough in the study population to trigger decreases in neutrophil degranulation. Indeed, evidence suggests that decreases in neutrophil capacity are associated with increasing neutrophilia (i.e. greater physiological stress) (Li and Cheng, 2007; Li and Gleeson, 2006). Exercise-induced changes in plasma hormones (adrenaline, cortisol, GH) and inflammatory mediators (IL-6, C5a) have been proposed to explain the decreases in neutrophil degranulation following prolonged exercise but the exact mechanisms remain unclear (Bishop et al., 2003; Henson et al., 1978; Laing et al., 2008; Pyne, 1994; Robson et al., 1999b; Yamada et al., 2002). The lack of measurement of these factors limits the discussion of the findings of the present study to previous studies.

To conclude, this is first to study to demonstrate that acute COL supplementation can enhance components of the innate immune system following prolonged exercise. It is proposed that this is primarily due to priming agents that become

bioavailable upon COL consumption. Further research is required to confirm this and identify whether a longer period of supplementation (to allow for greater absorption of priming components) has further benefits.

Chapter 4. Study 2 - Effect of 4 weeks of bovine colostrum supplementation on blood neutrophil responses and mucosal immunity following prolonged exercise

Abstract

Acute COL supplementation enhances neutrophil function and sLys concentration within a model of exercise-induced immune dysfunction. The aims of this study were to identify the effects of 4 weeks of COL supplementation on neutrophil responses and mucosal immunity following prolonged exercise. Participants were randomly assigned to 20 g per day of COL (n = 10, age: 28.8 ± 7.6 years, BM: 79 ± 8 kg, height: 183 ± 6 cm, $\dot{V}O_2$ max 54.3 ± 8.6 mL·kg^{-1}·min^{-1}) or PLA (n = 10, age: 27 ± 9 years, BM: 78.0 ± 7.7 kg, height: 180 ± 7 cm, $\dot{V}O_2$ max 55.7 ± 8.8 mL kg^{-1}·min^{-1}) for 4 weeks. Venous blood and unstimulated saliva samples were obtained before and after 2.5 h of cycling at 15% Δ (~ 55-60% VO_2 max). A significantly greater fMLP-stimulated oxidative burst was observed in COL group compared with PLA group ($p < 0.05$) and a trend toward a trial × time interaction ($p = 0.06$). However, there was no effect of COL on leukocyte trafficking, PMA-stimulated oxidative burst, bacterial-stimulated neutrophil degranulation, salivary SIgA, sLac or sLys ($p > 0.05$). These findings provide further evidence of the beneficial effects of COL on receptor-mediated stimulation of neutrophil oxidative burst in a model of exercise-induced immune dysfunction.

4.1 Introduction

Study 1 demonstrated that acute consumption of COL resulted in a greater fMLP-stimulated neutrophil oxidative burst and enhanced bacterial-stimulated neutrophil degranulation following prolonged exercise. This supported previous evidence from *in vitro* studies where short term co-culture with COL enhances oxidative burst activity of neutrophils. Sugisawa et al. (2003) proposed that in the presence of COL, PMN cells (i.e. neutrophils) become primed for subsequent activation by low-molecular weight substances (< 10 kDa) (such as protease peptones). Study 1 suggested that upon digestion of COL, these bioactive components or yet to be identified metabolites of COL become biologically available to prime the activity of neutrophils *in vivo*.

The magnitude of effects of COL on post-exercise stimulated-elastase release per neutrophil was similar to that reported in a 4 week study by Davison and Diment (2010). As neutrophil oxidative burst was not investigated in this 4 week study, the effects of longer term COL supplementation on this immune parameter in a prolonged exercise model remains unclear. If the above proposed mechanism is partly responsible for the effects of COL on immune function it may be hypothesised that 4 weeks of COL supplementation (longer period to allow for full absorption of COL and exposure to priming agents) would lead to a greater enhancement of oxidative burst.

In a similar way to blood neutrophil function, acute supplementation of COL showed beneficial effects on sLys (component of neutrophil granules) concentration post-exercise. There was, however, no effect of acute COL supplementation on sLac and salivary SIgA. Davison and Diment (2010) reported beneficial effects of 4 weeks of

COL supplementation on sLys concentration and secretion following prolonged exercise but observed no improvements in salivary SIgA. As sLac along with sLys are the most abundant AMPs in saliva, recognised to be important mediators of host defence (West et al., 2006, 2010), investigation into the effects of longer term supplementation on sLac is warranted. Davison and Diment (2010) suggested that longer term supplementation (e.g. 12 weeks) of COL or shorter durations (2 weeks) in multiple dosages across the day may lead to changes in resting concentrations of salivary SIgA. Enhancement of mucosal defences at rest (Crooks et al., 2006; Mero et al., 2002) or following exercise (Davison and Diment, 2010) could reduce the magnitude of any immunodepressive effects and the susceptibility to URI at the sites where most pathogens enter the body (Macpherson et al., 2012). Therefore, the aims of this study were to identify the effects of 4 weeks of COL supplementation on neutrophil responses and mucosal immunity following a similar prolonged exercise bout of study 1.

4.2 Methods

4.2.1 Participants

Twenty healthy, recreationally active males (age 28 ± 8 years; BM 78.6 ± 7.0 kg; height 182 ± 6 cm; $\dot{V}O_2$ max 55.0 ± 8.5 mL·kg^{-1}·min^{-1}) volunteered to participate in this investigation.

4.2.2 Supplementation

Participants were randomly assigned to a COL group (n = 10, age: 28.8 ± 7.6 years, BM: 79 ± 8 kg, height: 183 ± 6 cm, $\dot{V}O_2$ max 54.3 ± 8.6 mL·kg^{-1}·min^{-1}) or a PLA group (n = 10, age: 27 ± 9 years, BM: 78.0 ± 7.7 kg, height: 180 ± 7 cm, $\dot{V}O_2$ max

55.7 ± 8.8 mL $kg^{-1} \cdot min^{-1}$). In a double blind manner, participants were asked to consume 20 g per day (split into a morning and evening 10 g dose on an empty stomach) of COL or an isoenergetic/isomacronutrient PLA for 4 weeks.

4.2.3 Experimental trials

Participants reported to the laboratory (following an overnight fast of at least 10 h) for the collection of a blood and saliva sample (BAS) prior to commencing the above supplementation. Following preliminary measurements ($\dot{V}O_2$max determination and familiarisation on day 8 and 15 of supplementation respectively) and 4 weeks of supplementation (28 days), participants completed the main experimental trial. During the 48 h preceding the experimental trial, participants were asked to refrain from heavy exercise and alcohol consumption.

On the morning of the experimental trial, participants reported to the laboratory after an overnight fast of at least 10 h and at the same time of day as their BAS visit. The participants were asked to consume 500 mL of water 2 h before arrival to encourage euhydration. Participants were asked to remain seated for 10 min prior to collection of a resting blood sample (PRE) from an antecubital vein and an unstimulated saliva sample. Following collection of samples, participants immediately commenced 2.5 h of cycling at 15% Δ. All participants were permitted diluted cordial (four volumes of water to 1 volume of sugar-free cordial at 2 $mL \cdot min^{-1}$ kg of BM) every 15 min during the exercise but not at end of the exercise.

Expired gas was analysed during the 30th, 60th, 90th and 120th min of exercise (Jaeger Oxycon Pro, Hoechberg, Germany). HR and RPE was monitored every 15 min during the protocol using a telemetric device (Polar S610, Polar Electro Oy,

Kempele, Finland) and Borg rating scale (Borg, 1982) respectively. The mean of each of the physiological responses were used to determine if similar physical stress was imposed in each trial. A venous blood sample and an unstimulated saliva sample were collected immediately post-exercise (POST). Participants remained fasted until a further blood and saliva sample was obtained at 1 h post-exercise (1h POST).

4.3 Results

4.3.1 Physiological variables

There was no significant difference in $\dot{V}O_2$ (p = 0.479; p = 0.445) between the COL (2406 ± 187 mL·min^{-1}; 56.7 ± 3.8 % $\dot{V}O_2$ max) and PLA trial (2497 ± 351 mL·min^{-1}; 58.0 ± 3.8 % $\dot{V}O_2$max). Mean HR of 129 ± 6 bpm and 137 ± 9 bpm during COL and PLA trials respectively, were significantly different (p = 0.03). There was no difference (p = 0.989) when HR was compared between COL (73 ± 2%) and PLA (74 ± 3%) trials as a proportion of peak HR obtained during $\dot{V}O_2$max determination. There was no significant difference in RPE (p = 0.158) between the COL (12.4 ± 1.9) and PLA (13.5 ± 1.2) trial.

Similar pattern of plasma volume changes were observed from PRE between trials: COL; POST (-6.4 ± 4.8%); 1h POST (-3.1 ± 6.0) and PLA; POST (-4.5 ± 2.7%), 1h POST (-1.7 ± 3.3%). As there was no significant difference between trials (trial x time interaction; p = 0.697), it was deemed unnecessary to correct any haematological parameters for plasma volume changes. Although there was a significant change across time (p = 0.004), there was no significant trial (p = 0.937) or interaction effect (p = 0.771) on plasma glucose (Table 4.1). Post hoc analysis revealed a significantly

lower plasma glucose at 1h POST compared to both PRE ($p < 0.001$) and POST ($p = 0.031$). There was also a significant change across time for plasma lactate ($p = 0.002$) but no effect of trial ($p = 0.796$) or interaction ($p = 0.751$) (Table 4.1). Plasma lactate significantly increased from PRE (p 0.014) to POST before decreasing towards resting levels at 1h POST ($p = 1.000$).

Table 4.1 Plasma glucose and lactate responses following 4 weeks of COL or PLA.

Measure, $mmol \cdot L^{-1}$	PRE	POST	1h POST	p values trial time interaction
Plasma glucose				0.937
COL	4.87 ± 0.34	4.54 ± 0.86	4.29 ± 0.46	0.004*
PLA	4.79 ± 0.31	4.59 ± 0.52	4.28 ± 0.33	0.771
Plasma lactate				0.796
COL	1.36 ± 0.28	1.93 ± 0.67	1.54 ± 0.56	0.002*
PLA	1.49 ± 0.46	2.01 ± 0.73	1.46 ± 0.28	0.751

*Significant main effect of time ($p < 0.01$).

4.3.2 Immune cell counts

No significant trial or interaction effects were evident for total or differential leukocyte counts (Table 4.2). A main effect of time ($p < 0.001$) was observed in all leukocyte counts (Table 4.2). There was a significant increase in total leukocytes, neutrophils, monocytes, neutrophil:lymphocyte ratio and large immature cells from timepoints pre-exercise (BAS and PRE) to POST ($p \leq 0.01$) with all of these counts also showing significant increases from BAS and PRE to 1h POST ($p < 0.01$). Total lymphocyte count significantly increased from BAS to POST ($p = 0.022$). There was a significant increase in neutrophils, neutrophil:lymphocyte ratio ($p < 0.001$) and decrease in total lymphocytes ($p < 0.001$) and monocytes ($p = 0.036$) from POST to 1h POST.

Table 4.2 Immune cell counts following 4 weeks of COL or PLA.

Cell count, $10^9 \cdot L^{-1}$	BAS	PRE	POST	1h POST	p values trial time interaction
Total leukocytes					0.528
COL	4.91 ± 1.10	5.14 ± 1.07	11.46 ± 5.73	11.13 ± 4.47	< 0.001*
PLA	5.33 ± 1.60	5.62 ± 1.37	11.95 ± 4.39	11.51 ± 3.62	0.965
Neutrophils					0.611
COL	2.57 ± 0.81	2.50 ± 1.28	7.84 ± 4.14	8.32 ± 3.81	< 0.001*
PLA	2.48 ± 0.79	2.78 ± 0.83	8.45 ± 3.74	8.78 ± 3.36	0.679
Monocytes					0.899
COL	0.43 ± 0.15	0.52 ± 0.12	0.95 ± 0.58	0.84 ± 0.43	< 0.001*
PLA	0.50 ± 0.23	0.54 ± 0.12	0.79 ± 0.22	0.72 ± 0.21	0.336
Total lymphocytes					0.622
COL	1.65 ± 0.46	1.90 ± 0.71	2.41 ± 1.12	1.77 ± 0.46	0.001*
PLA	2.02 ± 0.68	2.02 ± 0.58	2.32 ± 0.57	1.69 ± 0.33	0.278
Neutrophil:lymphocyte					0.883
COL	1.61 ± 0.61	1.45 ± 0.72	3.36 ± 1.21	4.74 ± 1.90	< 0.001*
PLA	1.32 ± 0.48	1.45 ± 0.50	3.70 ± 1.46	5.35 ± 2.33	0.176
Large immature cells					0.679
COL	0.03 ± 0.02	0.04 ± 0.01	0.20 ± 0.23	0.19 ± 0.17	< 0.001*
PLA	0.04 ± 0.03	0.05 ± 0.02	0.16 ± 0.09	0.15 ± 0.05	0.598

*Significant main effect of time ($p < 0.001$).

4.3.3 Neutrophil responses

A significant main effect time of time ($p < 0.001$) was observed for fMLP-stimulated CL per neutrophil (neutrophil oxidative burst) (Figure 4.1A). There was a significant decrease in fMLP-stimulated CL per neutrophil compared with PRE at POST ($p = 0.001$) and 1h POST ($p < 0.001$). There was a main effect of trial for fMLP-stimulated CL per neutrophil ($p = 0.049$) and an interaction effect (trial ^{x} time) which approached significance ($p = 0.060$, Figure 4.1A).

A significant main effect of time ($p < 0.001$) was observed for PMA-stimulated CL per neutrophil (neutrophil oxidative burst) (Figure 4.1B). There was a significant decrease in PMA-stimulated CL per neutrophil compared with PRE at POST ($p < 0.001$) and 1h POST ($p = 0.001$). There was a significant increase in PMA-stimulated CL per neutrophil from POST to 1h POST ($p = 0.014$). There was no main effect of trial ($p = 0.395$) or trial ^{x} time interaction ($p = 0.464$) for PMA-stimulated CL per neutrophil (Figure 4.1B). Two-way ANOVA on PMA-stimulated CL per neutrophil following fMLP stimulation showed a main effect of time ($p = 0.001$) but no effect of trial ($p = 0.152$) or interaction ($p = 0.135$) (Table 4.3). There was a significant decrease in PMA-stimulated CL per neutrophil following fMLP compared to PRE at POST ($p < 0.001$) and 1h POST ($p < 0.001$) with an increase towards resting levels from POST to 1h POST ($p < 0.001$).

A significant main effect of time ($p < 0.001$) was observed for unstimulated CL per neutrophil (basal ROS) (Table 4.3). There was a significant decrease in unstimulated CL per neutrophil compared to PRE at POST ($p < 0.001$) and 1h POST ($p = 0.001$). There was a significant decrease in unstimulated CL per neutrophil from POST to 1h POST ($p = 0.004$). There was no main effect of trial ($p = 0.842$) or trial ^{x} time interaction ($p = 0.940$) for unstimulated CL per neutrophil (Table 4.3). A significant main effect of time ($p < 0.001$) was observed for $CL \cdot L^{-1}$ blood (Table 4.3). There was a significant increase in spontaneous $CL \cdot L^{-1}$ blood compared to PRE at POST ($p < 0.001$) and 1h POST ($p = 0.001$). Spontaneous $CL \cdot L^{-1}$ blood significantly decreased from POST to 1h POST ($p = 0.014$). There was no main effect of trial ($p = 0.071$) or trial ^{x} time interaction ($p = 0.539$) for spontaneous $CL \cdot L^{-1}$ blood (Table 4.3). There was no significant main effect of time ($p = 0.629$) trial ($p = 0.538$) or trial ^{x} time interaction ($p = 0.687$) for stimulated elastase release per neutrophil (neutrophil degranulation) (Table 4.3).

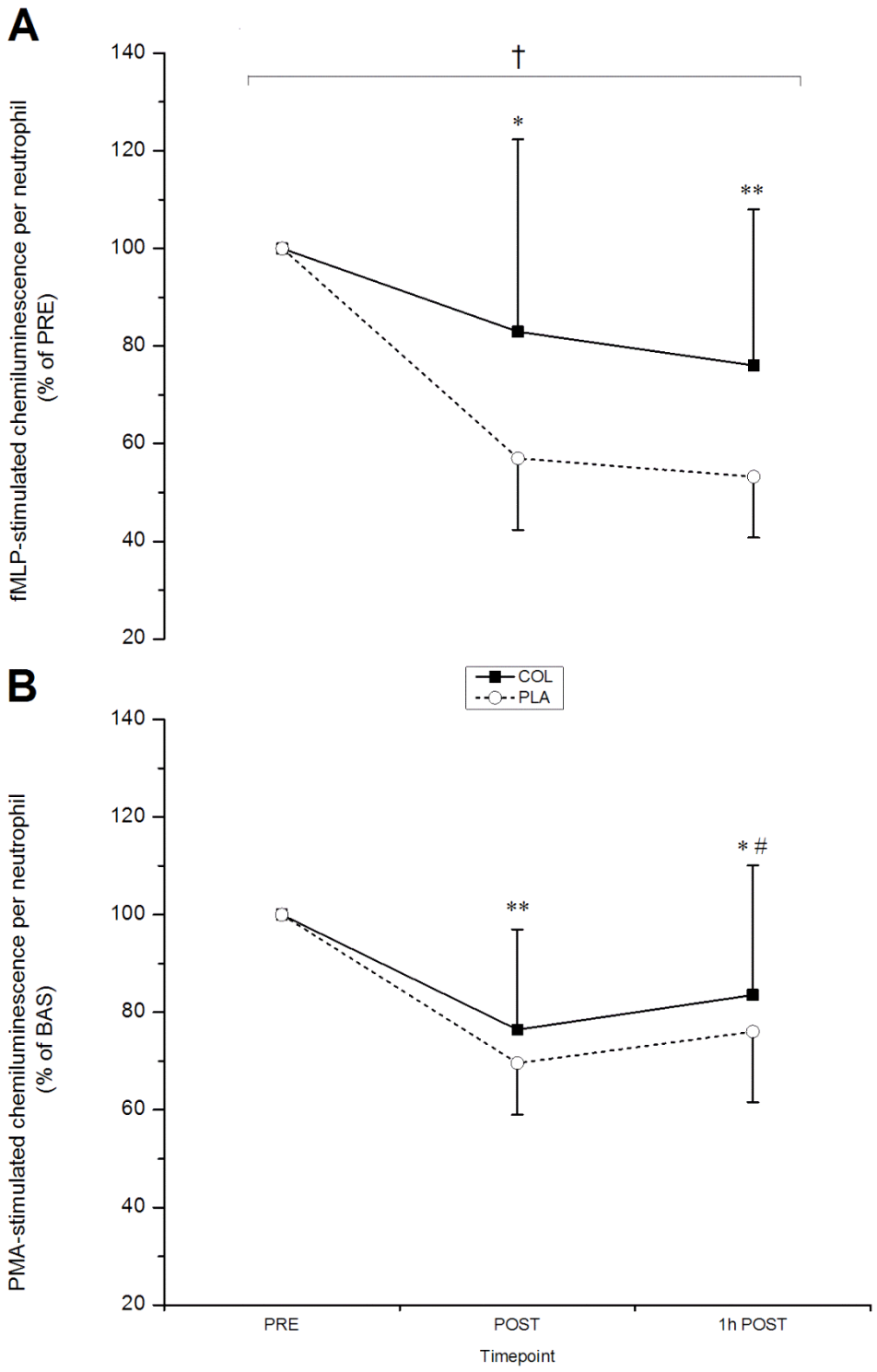

Figure 4.1 fMLP (A) and PMA (B) stimulated chemiluminescence per neutrophil following 4 weeks of COL or PLA. Significant change from PRE: *p = 0.001, **p < 0.001. #Significant change from POST (p < 0.05). †Significant main effect of trial for fMLP-stimulated chemiluminescence per neutrophil (p < 0.05).

Table 4.3 Neutrophil degranulation and oxidative responses following 4 weeks of COL or PLA..

Measure	PRE	POST	1h POST	p values trial time interaction
PMA-stimulated CL (oxidative burst) following fMLP stimulation (% of PRE)				0.152
COL	100 ± 0	61.9 ± 16.9	71.6 ± 16.2	0.001*
PLA	100 ± 0	51.6 ± 18.3	58.6 ± 16.2	0.135
Unstimulated CL per neutrophil (% of PRE)				0.842.
COL	100 ± 0	69.0 ± 45.9	57.2 ± 36.9	< 0.001**
PLA	100 ± 0	63.1. ± 31.9	54.0 ± 29.2	0.940
Spontaneous $CL \cdot L^{-1}$ blood				0.071
COL	813 ± 193	1110 ± 323	1042 ± 326	< 0.001**
PLA	1114 ± 335	1502 ± 537	1309 ± 467	0.539
Stimulated elastase release per neutrophil ($fg \cdot cell^{-1}$)				0.538
COL	369 ± 85	428 ± 310	374 ± 159	0.629
PLA	444 ± 144	396 ± 166	447 ± 233	0.687

Significant main effect of time (*p < 0.01, **p < 0.001).

4.3.4 Saliva responses

There was no significant main effect of time (p =0.903), trial (p = 0.801) or interaction (p = 0.773) for salivary SIgA concentration (Table 4.4). Two-way ANOVA revealed no significant main effect of time (p = 0.430) trial (p = 0.845) or interaction (p = 0.159) for salivary SIgA secretion rate (Table 4.4). There was no main effect of trial (p = 0.865) or interaction (p = 0.550) for saliva SIgA:osmolality (Table 4.4). There was, however, a main effect of time (p < 0.001) with post hoc analysis showing a significantly decreased saliva SIgA:osmolality from BAS and PRE to POST (p < 0.001) and an increase towards resting levels from POST to 1h POST (p = 0.022).

Table 4.4 Salivary SIgA responses following 4 weeks of COL or PLA.

Immune measure	BAS	PRE	POST	1h POST	*p* value trial time interaction
SIgA concentration ($mg \cdot L^{-1}$)					0.933
COL	336 ± 91	318 ± 91	321 ± 104	323 ± 100	0.801
PLA	325 ± 101	339 ± 115	345 ± 85	332 ± 94	0.773
SIgA secretion rate ($\mu g \cdot min^{-1}$)					0.845
COL	169 ± 77	141 ± 69	163 ± 97	186 ± 102	0.430
PLA	155 ± 99	180 ± 96	179 ± 92	174 ± 91	0.159
SIgA:osmolality ($mg \cdot mosmol^{-1}$)					0.865
COL	5.1 ± 1.0	5.1 ± 1.2	3.9 ± 1.2	4.7 ± 1.7	< 0.001*
PLA	5.6 ± 1.7	5.4 ± 1.0	3.9 ± 1.3	5.0 ± 1.3	0.550

*Significant main effect of time ($p < 0.001$).

There was no main effect or trial ($p = 0.195$) or interaction ($p = 0.244$) for sLac concentration (Table 4.5). There was a significant main time effect ($p < 0.001$) with post hoc analysis showing that sLac concentration was increased at POST compared to BAS ($p < 0.001$) and PRE ($p = 0.002$) followed by a decrease from POST to 1 h ($p = 0.005$). Two way ANOVA also revealed a main effect of time for sLac secretion rate ($p < 0.001$) (Table 4.5). Post hoc analysis identified greater sLys secretion rate at POST ($p < 0.001$) and 1h POST ($p = 0.030$) compared to BAS as well as an increase from PRE to POST ($p =0.026$). Results for sLac:osmolality showed no effect of time ($p = 0.405$), trial ($p = 0.448$) or interaction ($p = 0.711$) (Table 4.5).

Two-way ANOVA showed a significant main effect of time for sLys concentration ($p = 0.003$) but no effect of trial ($p = 0. 359$) or interaction ($p = 0.386$) (Table 4.5). Post

hoc analysis revealed an increase in sLys concentration from BAS to POST (p = 0.012). There was a main effect of time (p = 0.009) for sLys secretion rate but no effect of trial (p = 0. 514) or interaction (p = 0.117) (Table 4.5). Post hoc analysis revealed an increase in sLys secretion rate from BAS to 1h POST (p = 0.023). Two-way ANOVA showed no effect of time (p = 0.127), trial (p = 0.392) or interaction (p = 0.663) for sLys:osmolality (Table 4.5).

Table 4.5 The responses of sLac and sLys following 4 weeks of COL or PLA.

Immune measure	BAS	PRE	POST	1h POST	*p* value trial time interaction
sLac concentration ($mg \cdot L^{-1}$)					0.195
COL	3.9 ± 1.2	4.5 ± 5.3	5.3 ± 0.7	4.3 ± 0.5	< 0.001**
PLA	4.0 ± 1.2	4.7 ± 1.0	5.8 ± 1.0	5.2 ± 1.0	0.244
sLac secretion rate ($\mu g \cdot min^{-1}$)					0.186
COL	2.1 ± 1.2	2.0 ± 1.3	2.7 ± 1.5	2.4 ± 0.8	< 0.001**
PLA	2.4 ± 1.3	3.0 ± 1.6	3.6 ± 1.6	3.4 ± 1.4	0.256
sLac:osmolality ($mg \cdot mosmol^{-1}$)					0.448
COL	0.03 ± 0.02	0.03 ± 0.03	0.03 ± 0.02	0.04 ± 0.02	0.405
PLA	0.04 ± 0.02	0.05 ± 0.02	0.05± 0.02	0.05 ± 0.02	0.711
sLys concentration ($mg \cdot L^{-1}$)					0.368
COL	13.7 ± 6.0	18.8 ± 14.7	21.5 ± 11.6	22.2 ± 13.6	0.003*
PLA	13.5 ± 5.4	20.4 ± 10.6	32.5 ± 12.6	23.7 ± 6.2	0.359
sLys secretion rate ($\mu g \cdot min^{-1}$)					0.514
COL	6.6 ± 4.8	6.8 ± 5.8	11.3 ± 7.6	10.3 ± 3.2	0.009*
PLA	6.9 ± 6.2	12.7 ± 11.0	20.0 ± 11.3	15.5 ± 6.1	0.117
sLys:osmolality ($mg \cdot mosmol^{-1}$)					0.392
COL	0.23 ± 0.13	0.33 ± 0.29	0.27 ± 0.14	0.35 ± 0.25	0.127
PLA	0.26 ± 0.14	0.40 ± 0.26	0.42 ± 0.14	0.37 ± 0.09	0.663

Significant main effect of time (*p < 0.01,** p < 0.001)

4.4 Discussion

The present study demonstrated that 4 weeks of COL supplementation results in greater fMLP-oxidative burst responses compared to PLA. Although these findings expand on the beneficial effect observed on fMLP-stimulated responses with acute COL supplementation (Study 1), previously reported effects of 4 weeks COL supplementation on other innate and mucosal parameters could not be replicated in this study (i.e. stimulated-elastase release, sLys enhancement) (Davison and Diment, 2010).

Similar to study 1 and other investigations of prolonged exercise, there were significant increases in leukocytes, neutrophils, lymphocytes, neutrophil:lymphocyte, monocytes and immature cells (Davison and Diment, 2010; Davison et al., 2012; Lancaster et al., 2003; Li and Gleeson, 2004, 2005; McCarthy and Dale, 1988; Nieman, 1998). As shown previously (Carol et al., 2011; Davison and Diment, 2010; Study 1), there were no differences between the COL and PLA groups for leukocyte trafficking. Taken together, evidence suggests that is likely the effects of COL on immune function within a stress model of prolonged exercise are not related to the attenuation of the perturbations in leukocytes that occur as a result of increases in catecholamine or HPA activation (e.g. leukocytosis) (McCarthy and Dale, 1988).

The findings of *in vitro* culture demonstrate that COL possesses the ability to prime neutrophil function (Benson et al., 2012; Sugisawa et al., 2001, 2002, 2003). Neutrophils can be primed by a range of cytokines (GM-CSF, G-CSF, IL-8, TNFα) which amplify responses to subsequent stimulation (Elbim et al., 1994; Khwaja et al., 1992; Mikami et al., 1998; You et al., 1991). Sugisawa et al. (2003) proposed that 'priming' induced by COL are due to low molecular weight proteins (e.g. proteose

peptones) other than cytokines. Although investigations into the role of cytokines in the effects of COL on human immune function are limited, the findings here and within study 1 suggest that consumption of COL does increase the bioavailability of components which enhance neutrophil oxidative activity to physiological agonists (fMLP). It is currently unclear how long any priming effect of COL lasts but it is becoming apparent that the effects occur with receptor-mediated stimulation of neutrophils. It has previously been reported that enhanced oxidative burst responses to fMLP can last at least 24 h following exposure to priming agents (e.g. G-CSF) (Ichinose et al., 1990). It is worthy to note that the findings from this study, and those of study 1, suggest that the mechanisms underlying the effects of COL are not related to up-regulation of antioxidant capacity or modulation of basal ROS production following exercise.

This study showed no attenuation of exercise-induced decreases in PMA-stimulated oxidative burst with or without prior fMLP stimulation, supporting evidence gathered from study 1. It may be suggested that COL may not exert effects on neutrophil responses via PKC and/or does not affect the maximal capacity of neutrophils following prolonged exercise. Although the activation of PKC has been shown to occur following stimulation by some physiological agonists (Peake, 2002), PMA is not encountered *in vivo* and hence considered as an artificial stimulus (Sheppard et al., 2005). Due to the irreversible nature of PMA stimulation, it differs substantially to physiological agonists (e.g. fMLP) (DeCoursey and Ligeti, 2005).The effectiveness of PMA to stimulate pathways for ROS production *in vitro* has perhaps masked the irrelevancy of the agonist towards *in vivo* neutrophil function (Sheppard et al., 2005). In contrast, production of formylated proteins (e.g. fMLP) is attributed to bacteria and mitochondria, where a receptor (FPR1) on the cell surface of neutrophils recognises

the microbial moieties and tissue damage (Jaillon et al., 2013; Zhang et al., 2010). Therefore, the potential *in vivo* significance of enhanced fMLP-induced oxidative burst by acute and longer term COL supplementation may be enhanced responses toward infectious and/or inflammatory challenge (Carp, 1982; Marasco et al., 1984; Tennenberg and Solomkin, 1990).

Despite the exercise-induced fall in neutrophil oxidative burst, there was no effect of prolonged exercise on neutrophil stimulated-elastase release. This is in contrast to findings reported by numerous investigators (Davison and Gleeson, 2005, 2006, 2006; Davison et al., 2007, 2012; Laing et al., 2005, 2008) but supports others (Li and Gleeson, 2005). It has been proposed that increasing neutrophilia following prolonged exercise may be associated with decreases in neutrophil function (Li and Cheng, 2007; Li and Gleeson, 2006). In general, neutrophilia observed in the studies where stimulated-elastase release was unaffected by exercise have not been to the same extent as where exercise-induced decreases have been found. Based on the available evidence, it is hypothetical to suggest that the exercise stress, in particular the exercise intensity in this study was not sufficient to induce decrements in neutrophil degranulation within the study population. The comparison of this study to others is limited by the lack of measurement in potential mediators but it is likely that the exercise intensity was not of an adequate strenuous nature to induce the elevations in circulating concentrations of adrenaline (with subsequent increases in intracellular cyclic adenosine monophosphate) cortisol, growth hormone, IL-6, and/or C5a to levels where perturbations in mobilisation and degranulation of neutrophils were seen previously (Bishop et al., 2003; Henson et al., 1978; Laing et al., 2008; Peake, 2002; Pyne, 1994; Robson et al., 1999b; Yamada et al., 2002). Nevertheless the findings of this study and our previous study (Study 1) may support the

hypothesis that oxidative burst capacity may be more susceptible to the duration rather than exercise intensity *per se* (Peake, 2002; Peake et al., 2004; Pyne et al., 2000; Robson et al., 1999b).

The absence of a fall in neutrophil degranulation (bacterial-stimulated elastase) in this study (hence a reduced scope for nutritional intervention) may also explain why there was a discrepancy to effects observed with 4 weeks of supplementation in Davison and Diment (2010). The azurophilic granules of neutrophils are also abundant in AMPs e.g. lysozyme (Amulic et al., 2012). In contrast, to Davison and Diment (2010) and study 1, there was also no enhancement in sLys within the present study. The two aforementioned studies observed a decline in neutrophil degranulation in the PLA groups during the recovery period. Although further research is required to ascertain the role of oral neutrophils in exercise-induced immune depression, changes in sLys (as other AMPs) may reflect the activity of circulating neutrophils (Davison et al., 2009). The large variability in sLys in the PLA group in this study is in accordance with others (Hopkins, 2000; West et al., 2010) who have recognised the variance as a source of limitation for detection of intervention induced-changes in mucosal parameters.

As shown previously (Gillum et al., 2013; Study 1), there was an increased availability of sLac following prolonged exercise in both COL and PLA trials. The reason for this increase remains unclear but may be indicative of an inflammatory response induced by damaged epithelial cells (Davison et al., 2009; Usui et al., 2011). Given that longitudinal studies of sLac have observed lower levels in athletes compared to resting controls (West et al., 2010), further investigations are required

to determine whether responses to prolonged exercise on a regular basis leads to a depletion of sLac availability.

Reduced mobilisation of salivary SIgA has been advocated as a marker of dysfunctional mucosal immunity following prolonged exercise (Walsh et al., 2011b). Despite no change in concentration or secretion, there was an exercise-induced decrease in saliva SIgA:osmolality. This reduction was unaltered by COL supplementation. COL has previously been shown to increase resting salivary SIgA concentrations (Crooks et al., 2006; Mero et al., 2002), but this was not evident in the present study. Based on Crooks et al. (2006) and Mero et al. (2002), Davison and Diment (2010) proposed that the dosage of COL may need to be split across the day or taken over longer periods to stimulate changes in resting salivary SIgA production. Although not separated into four doses (5 g) as Mero et al. (2002), the present study did split the COL dosage into a morning and evening dose of 10 g. The variance in results may be explained by the composition of the COL and the assay used in the determination of IgA. Further evidence is required to confirm the findings of Crooks et al. (2006) that significant changes in salivary SIgA are only evident after 12 weeks of supplementation.

In conclusion, the primary finding of the study was that COL supplementation leads to a greater fMLP-oxidative burst than a PLA group. There was, however, no effect of COL on all other parameters of innate and mucosal immunity. Further research is required to elucidate the mechanisms underlying COL supplementation and whether beneficial effects of COL may become apparent at rest when consumed over longer periods.

Chapter 5. Study 3 - Effects of bovine colostrum supplementation on upper respiratory illness in active males

Abstract

COL has been advocated as a nutritional countermeasure to exercise-induced immune dysfunction and increased risk of URI in athletic populations, however, the mechanisms remain unclear. During winter months, under double-blind procedures, 53 males (mean training load ± SD, 50.5 ± 28.9 MET-h week^{-1}) were randomized to daily supplementation of 20 g of COL (n = 25) or an isoenergetic/isomacronutrient PLA (n = 28) for 12 weeks. Venous blood was collected at baseline and at 12 weeks and unstimulated saliva samples at 4 weeks intervals. There was a significantly lower proportion of URI days and number of URI episodes with COL compared to PLA over the 12 weeks ($p < 0.05$). There was no effect of COL on in vitro neutrophil oxidative burst, salivary SIgA or AMPs ($p > 0.05$), which does not support previously suggested mechanisms. In a subset of participants (COL = 14, PLA = 17), real-time quantitative PCR, targeting the 16S rRNA gene showed there was an increase in salivary bacterial load over the 12 weeks period with PLA ($p < 0.05$) which was not as evident with COL. Discriminant function analysis of outputs received from serum metabolomics showed changes across time but not between groups. This is the first study to demonstrate that COL limits the increased salivary bacterial load in physically active males during the winter months which may provide a novel mechanism of immune-modulation with COL and a relevant marker of in vivo (innate) immunity and risk of URI.

5.1 Introduction

It is now well established that exercise of a strenuous and/or prolonged nature can lead to significant transient perturbations of immune function (commonly referred to as immunodepression) which includes, but is not limited to, decreases in both cell-mediated and mucosal parameters (Nieman, 2007). This may result in an 'open window' during which risk of illness is increased (Nieman, 2000). Hence, if such exercise is performed on a regular basis, as with endurance athletes, and particularly in combination with other life stressors (e.g. inadequate nutrition, psychological stress) the overall risk can be substantially higher (Gleeson, 2007). The increase in the frequency and severity of symptoms of URI in athletes (e.g. sore throat, runny nose) has been attributed to such periods of heavy exertion (Walsh et al., 2011b).

COL may be effective at alleviating recurrent URI in situations of immune deficiency (Patel and Rana, 2006). Previous evidence has shown that 8-10 weeks of COL supplementation can reduce the incidence of URI in physically active populations but the mechanism(s) behind such effects remains unclear (Brinkworth and Buckley, 2003; Crooks et al., 2010). Animal and *in vitro* culture studies demonstrate that COL has a mediating effect on cell-mediated immunity by influencing the production of cytokines (Biswas et al., 2007; Shing et al., 2009b). Increasing concentrations of COL, *in vitro*, has been shown to modulate cytokine production in peripheral blood mononuclear cells from resting, healthy individuals, to promote a Th1 profile (cell-mediated immunity) (Shing et al., 2009b), which may suppress the binding of pathogens (e.g. rhinovirus) (Sethi et al., 1997). Direct effects of COL on leukocyte capacity are also supported by evidence of an enhancement of phagocytosis and oxidative burst of PMN cells (i.e. neutrophils) following short term culture with COL

(Sugisawa et al., 2001, 2002, 2003). Sugisawa et al. (2003) proposed that in the presence of COL leukocytes become primed for subsequent activation by low-molecular weight substances (< 10 kDa) such as protease peptones.

Given the aforementioned effects of COL within inflammatory *in vitro* culture conditions, it may be expected that COL can act as a nutritional countermeasure to exercise-induced immunodepression. Our previous work suggests that 4 weeks of COL (20 $g \cdot day^{-1}$) supplementation can limit the immunodepressive effects of an acute physical stressor (2 h of cycling) by enhancing neutrophil function (stimulated degranulation/elastase release) post-exercise (Davison and Diment, 2010). Within the study the modulatory effects of COL also extended to innate mucosal immunity by preventing the exercise-induced decrease of sLys concentration and secretion.

Study 1 also demonstrated the immune enhancing potential of acute COL supplementation on innate immunity (stimulated degranulation, fMLP-stimulated oxidative burst; sLys concentration). Such findings may provide support to proposed mechanisms that some of the immune-modulatory effects of COL are due to bioactive components that become biologically available upon digestion of COL and prime leukocyte capacity (Davison, 2013). It is currently unclear whether longer term supplementation of COL and exposure to these priming agents or metabolites also leads to changes in innate markers in athletes at rest. Crooks et al. (2006) demonstrated that longer periods of COL supplementation (i.e. 12 weeks) may be associated with significant increases in resting concentrations of salivary SIgA, which is the only immune measure to date that has been consistently related to risk of URI in exercising populations (Walsh et al., 2011b). Other studies have also seen improvements in resting salivary SIgA concentrations with COL supplementation but

have not monitored URI (Appukutty et al., 2010; Mero et al., 2002). To date, the majority of both longitudinal and cross-sectional exercise training studies have focused on changes in salivary SIgA (Walsh et al., 2011b). Although the importance of other salivary AMPs (e.g. lysozyme, lactoferrin) for host defense have been recognised, they have received limited attention (West, 2006).

In addition to the presence of inducible factors such as AMPs at mucosal surfaces, protection from invading microorganisms is also provided by the diverse community of commensal microbes which colonise the upper respiratory tract (Blaser and Falkow, 2009; Bosch et al., 2013). Subsequently, disturbance of this respiratory microbial community can contribute to acquisition of new pathogens which may result in respiratory illness, particularly if host immunity is compromised (Murphy et al., 2009). However, the effects of exercise and nutritional interventions on changes in the salivary microbiome have not previously been investigated.

The aims of this study were to investigate the effects of 12 weeks of COL supplementation on innate and mucosal immunity as well as the salivary microbiome in a population of males who engage in exercise training during the winter months. The study also aimed to determine whether any effects of COL on these parameters would also lead to a change in the incidence of URI. Given the potential involvement of a diverse array of biological pathways, we also undertook a metabolomic profiling approach on serum in an attempt to gain a more detailed understanding of any modulation of the immune system by COL.

5.2 Methods

5.2.1 Participants

Fifty seven males, completing at least 3 h of moderate-vigorous endurance exercise a week and free of influenza vaccinations in the previous 12 months, volunteered to participate in the present investigation.

5.2.2 Supplementation

All 57 participants were randomly allocated into COL or PLA groups with stratification by age and type of exercise training only. In a double blind manner, participants were asked to consume 20 g·day^{-1} (10 g prior to morning and evening meal) of COL or an isoenergetic/isomacronutrient PLA for 12 weeks. Four participants (COL = 3, PLA = 1) were lost due to lack of compliance with the study protocol (e.g. lack of training or supplement consumption due to injury, family bereavement or air travel). All participants who successfully completed the study (COL group, n = 25, age: 30.5 ± 13.8 years, BM: 77.2 ± 8.9 kg, height: 179.9 ± 6.4 cm); PLA group, n = 28, age: 31.5 ± 13.2 years, BM 74.5 ± 8.7 kg, height 178.4 ± 6.6 cm) commenced the study between September and December.

5.2.3 Serum Metabolomics

Blood samples were collected at baseline and at 12 weeks following COL or PLA supplementation. Serum obtained from all samples were analysed using Direct Injection Electrospray Ionisation Mass-Spectrometry (DI-ESI-MS) by colleagues (see acknowledgements section) at the Institute of Biological, Environmental and Rural Sciences. To remove proteins before mass spectrometry, 20 µL of each serum sample was mixed with 30 µL of ice-cold ultrapure water and vortexed. 50 µL of ice-

cold HPLC grade acetone (Fisher Scientific UK Ltd, Loughborough, UK) was then added and the mixture vortexed. Samples were left on ice for 30 min to allow protein precipitation, after which, they were centrifuged at 16000 *g* for 10 min in a micro-centrifuge. After centrifugation, 50 µL of the supernatant was removed and transferred to a glass insert placed in a glass auto-sampling vial, to which 250 µL of ice-cold 70% (v/v) methanol (made up using HPLC grade methanol (Fisher Scientific UK Ltd) and ultrapure water) was added. Seven control serum samples, from human male AB plasma (Sigma-Aldrich, Dorset, UK), were run simultaneously using the same protocol to act as machine and protocol controls.

Analysis (DI-ESI-MS) was carried out using on a Micromass LCT mass-spectrometer (Micromass/Waters Ltd., UK) in negative ionisation mode where metabolites are singly ionised by the loss of H^+. The polar extracts were reconstituted in 0.25 mL 30% [v/v] methanol: H_2O and 50 µL added to 200 µL inserts in 2 mL (Waters Ltd. UK) and introduced by direct-infusion (DI) at a flow rate of 0.05 mL min^{-1} in 30% [v/v] methanol: H_2O running solvent. Data were acquired over the *m/z* range 100-1400 Th and were imported into MATLAB, binned to unit mass and then normalised to percentage total ion as stated in Johnson et al. (2007). Tentative identification of metabolites of interest was achieved through interrogation of the HMDB Serum Metabolome database (Psychogios et al., 2011).

5.2.4 Saliva sampling

Participants reported to the laboratory for a saliva sample at baseline and 4 weeks, 8 weeks and 12 weeks following supplementation. All participants avoided strenuous exercise 24 h prior to each visit and arrived at the laboratory after an overnight fast

of at least 10 h. Following centrifugation of saliva samples at baseline, 4 weeks, 8 weeks and 12 weeks, saliva supernatants (following centrifugation) were analysed for salivary SIgA and AMPs (detailed in sections 2.6.2 – 2.6.4). At baseline and at 12 weeks in 31 participants (COL = 14, PLA = 17), the saliva pellets (from saliva centrifugation) were used for DNA extraction.

5.2.5 Extraction of microbial DNA

Microbial DNA was extracted from 200 µL of the salivary pellet at baseline and 12 weeks of 31 participants who all commenced the study late September/early October and completed the study late December/early January. Extraction was performed using a FastDNA SPIN Kit (MP Biomedical, Santa Ana, USA) following the manufacturer's guidelines, except that bead beating was carried out using a FastPrep24 (MP Biomedical) machine with three cycles at speed setting 6.0 for 30 s, with cooling on ice for 60 s between each cycle. Extracted DNA was quantified using Epoch (BioTek, Winooski, USA) spectrophotometry. All extractions were confirmed to have a 260/280 nm ratio of between 1.8 and 2.0 for quality control purposes.

5.2.6 16S rRNA Gene Terminal Restriction Fragment Length Polymorphisms (T-RFLP)

Amplification of the 16S rRNA gene was accomplished through PCR using primers 27*f* (5'-AGA GTT TGA TCC TGG CTC AG-3' with FAM labelled on 5' end) and 1389*r* (5'-ACG GGC GGT GTG TAC AAG-3') as described by Huws et al. (2011). All PCR products were verified using a 1.0% agarose gel. Triplicate reactions for each sample were pooled and purified using a QIAquick PCR purification kit (Qiagen, West Sussex, UK) following the manufacturer's guidelines. 25 ng of purified PCR product for each sample was digested for 5 h at 37°C with restriction enzymes *Hae*III

and *Msp*I (Promega, Madison, USA), in separate reactions. Restriction products were separated through size using an ABI PRISM1 377 Automated DNA Sequencer (Applied Biosystems, Warrington, UK). As there was an inability to sequence the 16S rRNA genes at one of the timepoints for 2 participants, peak profiles and Shannon Diversity Indexes were determined on 29 participants only (COL = 12, PLA = 17). Peak profiles were examined using Genemapper software (Version 3.7, Applied Biosystems). Those peaks with an estimated fragment size below 50 nucleotides were removed and the remaining data were modelled using FingerPrinting II software (BioRad, Hercules, USA). Shannon Diversity Indexes were determined using Fingerprint Analysis with Missing Data software (Version 1.2) (Schluter and Harris, 2006).

5.2.7 16S rRNA Gene Quantitative PCR

Quantitative PCR was carried out on neat extracted DNA against standards created by amplifying the 16S rRNA gene of 5 randomly selected baseline samples. This used 1 µL of each sample in a PCR reaction using 27*f* and 1389*r* primers, as detailed above, except that the 27*f* primer did not have FAM on the 5' end, to amplify the gene. The resulting PCR product was purified and quantified, as previously detailed, to estimate the total number of 16S rRNA gene copies and serial dilutions made to a 10^{-10} level. Serial dilutions of 10^{-0}, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, and 10^{-10} were used in subsequent qPCR reactions using a C100 thermal cycler (BioRad) and CFX96 optical detector (BioRad), with data captured using CFX Manager software (BioRad). qPCR reactions were completed in 25 µL volumes consisting of 1X SYBR Green Mastermix (Applied Biosystems), 400 nM of each of the EubF forward (5'-GTG STG CAY GGT TGT CGT CA-3') and EubR reverse (5'-ACG TCR TCC MCA

CCT TCC TC-3') primers, as detailed by Kim et al. (2008) and 3 µL of neat DNA. The final volume was made up with PCR grade water (Roche, Hertfordshire, UK).

5.2.8 Microbial Growth Curve Analysis

To ascertain the antimicrobial properties of each supplement**,** 10 µL of a 50 $g \cdot L^{-1}$ (w/v) solution of COL or PLA (made using autoclaved ultrapure water), were cultured with 200 µL of artificial saliva medium and 10 µL of a salivary microbial culture as previously described by McBain et al. (2003). In addition, 10 µL of the COL and PLA solutions were incubated with 200 µL of artificial saliva medium, without the addition of the salivary microbial culture, to determine the level of microbial load for each solution. These cultures, alongside appropriate positive and negative growth controls, were incubated in a CellStar tissue culture 96 well plate with flat bottom and lid (Greiner Bio-one, Nürtingen, Germany) in a BioTek ELx808 microplate reader (BioTek Instruments, Winooski, USA) set at 37°C for 72 h. An optical density reading was taken every 20 min at a 630 nm wavelength, before which, the plate was shaken for 5 s.

Kinetic read data was exported from the Gen5 software package (BioTek Instruments) and corrected to a baseline (the first reading taken for each of the 96 wells). To allow for log transformation of the raw data, all data points, after baseline correction, were added to 10. The Log_{10} value for each data point was then calculated. After 3 replicate 96 well plates were completed, the mean and standard deviation for each growth condition, across all 3 plates, was calculated

5.2.9 Statistical analysis

Initially, a 2 factor mixed model ANOVA (group × time) was carried out on all immunological measures,16S rRNA (salivary bacterial load) and total peak number for *Hae*III and *Msp*I (salivary bacterial diversity) to determine if the effect of time was different between COL or PLA groups. Any significant main effects identified in the ANOVA were further analysed by post hoc paired t-tests with Holm-Bonferroni correction. Independent t-tests were used to determine differences between groups at baseline and 12 weeks in Shannon Diversity Indexes for TFRLP (*Hae*III *and Msp*I). Data for the proportion of reported URI days and proportion of participants who suffered URI over the 12 weeks between the COL and PLA groups were assessed by chi-squared test. To also examine the time-course of any effect of COL, chi-squared analyses of URI at 4 week intervals were performed, in accordance with the timing of saliva collections in the current study and the methods of Crooks et al. (2006, 2010). Chi-squared analysis was also used to assess proportion of participants within COL or PLA who reported use of medication during URI episodes. Comparisons between COL and PLA groups for mean number of self-reported URI episodes, mean duration of URI, mean severity of URI and mean weekly training loads were performed with an independent t-test. Metabolite data was analysed through multivariate statistics, including principal component analysis and discriminant function analysis (DFA), using PyChem software (Jarvis et al., 2006) and following accepted Metabolomics Standard Initiative procedures (Sansone et al., 2007). Those whole mass unit bins which had a DFA loading of more than 2 standard deviations from the mean were selected for tentative identification. Determination of change from baseline for each whole mass unit bins was determined using 1-way ANOVA.

5.3 Results

5.3.1 Training loads

Analysis of IPAQ questionnaires showed no significant differences between COL (53.1 ± 6.0 MET-h·week^{-1}) and PLA (48.2 ± 5.4 MET-h·week^{-1}) groups for weekly training volume at moderate-vigorous intensity ($p = 0.546$).

5.3.2 Upper respiratory illness

Chi-squared analysis showed a significantly lower proportion of days with URI during the 12 weeks in the COL group (5%) compared to the PLA group (9%) ($p < 0.001$). Further chi-squared analyses of URI at 4 week intervals showed a significantly lower proportion of days with URI in the COL group at 1-4 weeks (COL = 6%, PLA = 8%; $p = 0.021$) and 5-8 weeks (COL = 2%, PLA = 13%; $p < 0.001$) but not 9-12 weeks (COL = 6%, PLA = 7%; $p = 0.348$) (Figure 5.1). Independent t-test showed a significantly lower mean number of URI episodes in the COL group compared to PLA group over the 12 weeks (COL, 0.4 ± 0.7; PLA, 0.8 ± 0.7; $p = 0.033$). The proportion of participants who reported URI during the study period (all 12 weeks combined) was not lower in the COL group (40%) than PLA group (64%) ($p = 0.067$). There were, however, a significantly lower proportion of participants who reported URI in the COL group (12%) compared to PLA (36%) at 5-8 weeks ($p = 0.044$) which was not evident at 1-4 weeks (COL = 16 %, PLA = 25%; $p = 0.322$) or 9-12 weeks (COL = 20%, PLA = 18%; $p = 0.559$) (Figure 5.1). When URI episodes were reported by participants, the severity (COL, 35.3 ± 26.9; PLA, 42.0 ± 27.4; $p = 0.250$) and duration of symptoms (COL, 7.9 ± 4.4 days; PLA, 9.9 ± 5.6 days; $p = 0.159$) were similar between groups. During episodes of URI, there were no differences between groups for the proportion of participants who used medication (COL = 50%, PLA =

78%; p = 0.139). All of the above patterns/effects were similar in the subset of participants used for microbiome analysis (see section 5.25).

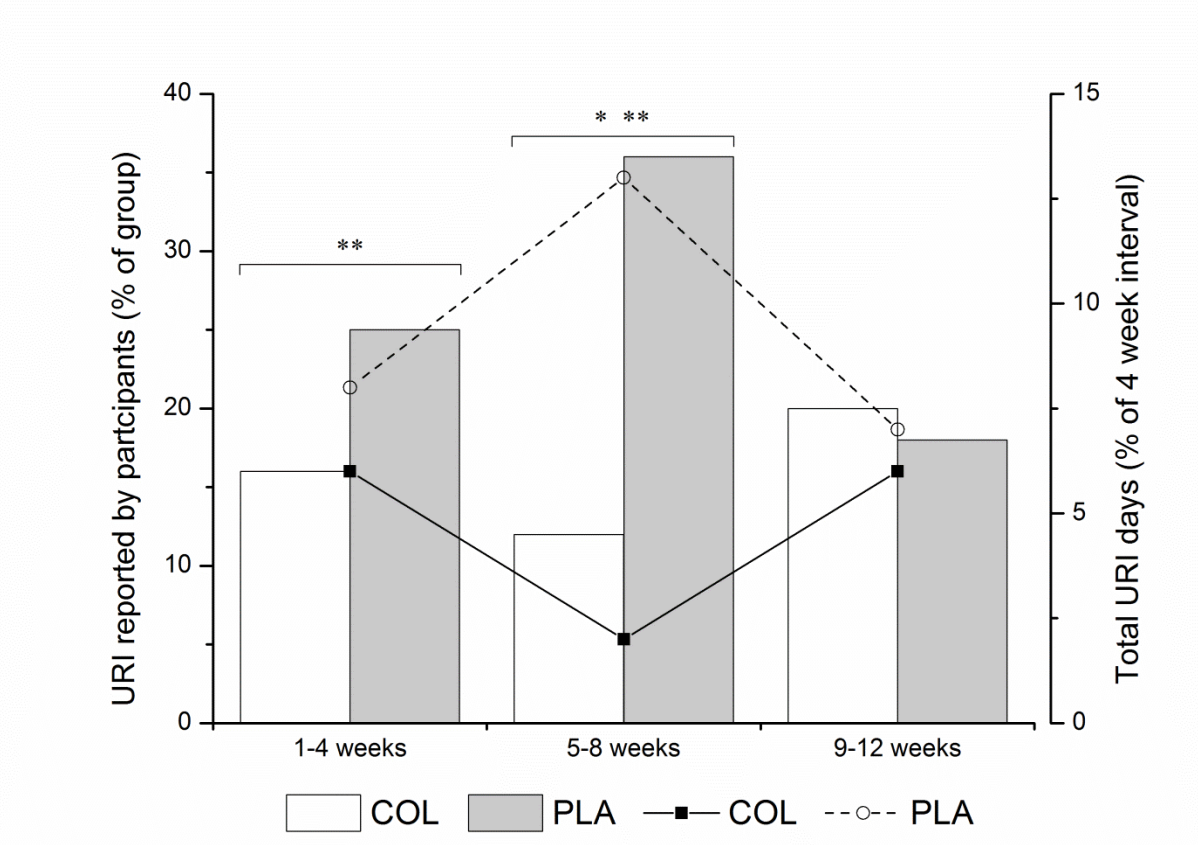

Figure 5.1 URI during 4 week intervals in COL and PLA groups. Columns reflect proportion of participants in each group who report URI. Line and symbol represent proportion of URI days in each group during 4 week intervals. **Significantly lower proportion of URI days in COL group at 1-4 weeks and 5-8 weeks ($p < 0.05$). *Significantly lower proportion of participants in the COL group who report URI at 5-8 weeks ($p < 0.05$).

5.3.3 Salivary SIgA and AMPs

The analyses of salivary AMPs across the 12 week period are shown in Table 5.1 and 5.2. One participant was removed from SIgA analysis due to a lack of clear supernatant upon centrifugation of saliva samples. Final comparison for salivary SIgA was conducted on 52 participants (COL = 25, PLA = 27). This participant had

been excluded from sLac and sLys for contamination purposes as previously mentioned (see above). There were no significant differences between groups in salivary measures during the 12 weeks ($p > 0.05$) (Table 5.1 and 5.2).

Table 5.1 Salivary SIgA before, during and at the end of 12 weeks of COL and PLA.

Immune measure	Baseline	4 weeks	8 weeks	12 weeks	p trial p time p interaction
SIgA concentration ($mg \cdot L^{-1}$)					0.286
COL	267 ± 127	253± 120	256 ± 143	272 ± 153	0.569
PLA	260 ± 203	217 ± 124	230 ± 132	215 ± 99	0.841
SIgA secretion rate ($\mu g \cdot min^{-1}$)					0.733
COL	117 ± 89	126 ± 106	130 ± 90	144 ± 97	0.065
PLA	121 ± 126	105 ± 52	114 ± 63	108 ± 57	0.192
SIgA:osmolality ($mg \cdot mOsmol^{-1}$)					0.280
COL	3.8 ± 1.5	3.6 ± 1.4	3.5 ± 1.4	3.8 ± 1.8	0.409
PLA	3.6 ± 2.0	3.19 ± 1.4	3.4 ± 1.5	3.1 ± 1.2	0.595

Table 5.2 sLac and sLys before, during and at end of 12 weeks of COL and PLA

Immune measure	BAS	4 weeks	8 weeks	12 weeks	p trial p time p interaction
sLac concentration ($mg \cdot L^{-1}$)					0.431
COL	3.3 ± 0.8	3.7 ± 0.8	3.8 ± 0.8	3.8 ± 0.8	0.032*
PLA	3.8 ± 1.2	3.8 ± 1.0	4.0 ± 1.0	3.8 ± 1.0	0.198
sLac secretion rate ($\mu g \cdot min^{-1}$)					0.365
COL	1.6 ± 1.0	1.9 ± 1.2	2.0 ± 0.9	2.1 ± 0.8	0.001**
PLA	1.9 ± 1.0	2.1 ± 1.2	2.3 ± 1.4	2.3 ± 1.3	0.829
sLac: osmolality ($mg \cdot mOsmol^{-1}$)					0.549
COL	0.05 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.111
PLA	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.06 ± 0.02	0.282
sLys concentration ($mg \cdot L^{-1}$)					0.837
COL	21.5 ± 13.3	23.0 ± 13.5	22.2 ± 12.6	20.1 ± 12.7	0.636
PLA	22.0 ± 14.6	20.1 ± 12.1	21.7 ± 11.5	21.1 ± 12.1	0.295
sLys secretion rate ($\mu g \cdot min^{-1}$)					0.764
COL	9.9 ± 8.3	10.5 ± 6.5	11.4 ± 8.4	10.0 ± 5.1	0.037*
PLA	10.8 ± 8.8	10.7 ± 8.4	11.7 ± 7.4	11.6 ± 8.8	0.627
sLys:osmolality ($mg \cdot mOsmol^{-1}$)					0.941
COL	0.3 ± 0.2	0.4 ± 0.3	0.3 ± 0.2	0.3 ± 0.2	0.622
PLA	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.420

Main effect of time indicated by * $p < 0.05$. Post hoc analysis could not reveal any significant differences between timepoints for sLac concentration. There was a significant increase in sLac secretion rate (at 8 and 12 weeks) and sLys secretion rate (at 8 weeks) from baseline ($p < 0.05$).

5.3.4 Salivary bacterial load and diversity

The ANOVA to analyse log of estimated copy number of 16S rRNA gene revealed a significant time ($p < 0.001$) and interaction effect ($p = 0.035$) but no group effect ($p = 0.602$) (Figure 5.2). Post hoc analysis of interaction revealed a significant increase in bacterial load over the 12 weeks in the PLA group ($p < .001$) that was not present in COL group ($p = 0.386$) (Figure 5.2). ANOVA on total TRFLP peak number obtained from the restriction enzyme *Msp*I revealed a main time effect ($p < 0.001$), with decreased bacterial diversity at 12 weeks, but no interaction ($p = 0.515$) or group effect ($p = 0.917$). However, analysis of total TRFLP peak number using *Hae*III revealed no time ($p = 0.123$), interaction ($p = 0.912$) or group effects ($p = 0.866$). Analysis of Shannon Diversity Indexes for TFRLP using *Msp*I showed no differences between groups at baseline ($p = 0.789$) or at 12 weeks ($p = 0.746$). There were also no differences at baseline ($p = 0.683$) or 12 weeks ($p = 0.823$) in Shannon Diversity Indexes for TFRLP using *Hae*III. Microbial growth analysis, showed no indication of a direct antimicrobial effect of either the COL or PLA supplement (see Appendix C).

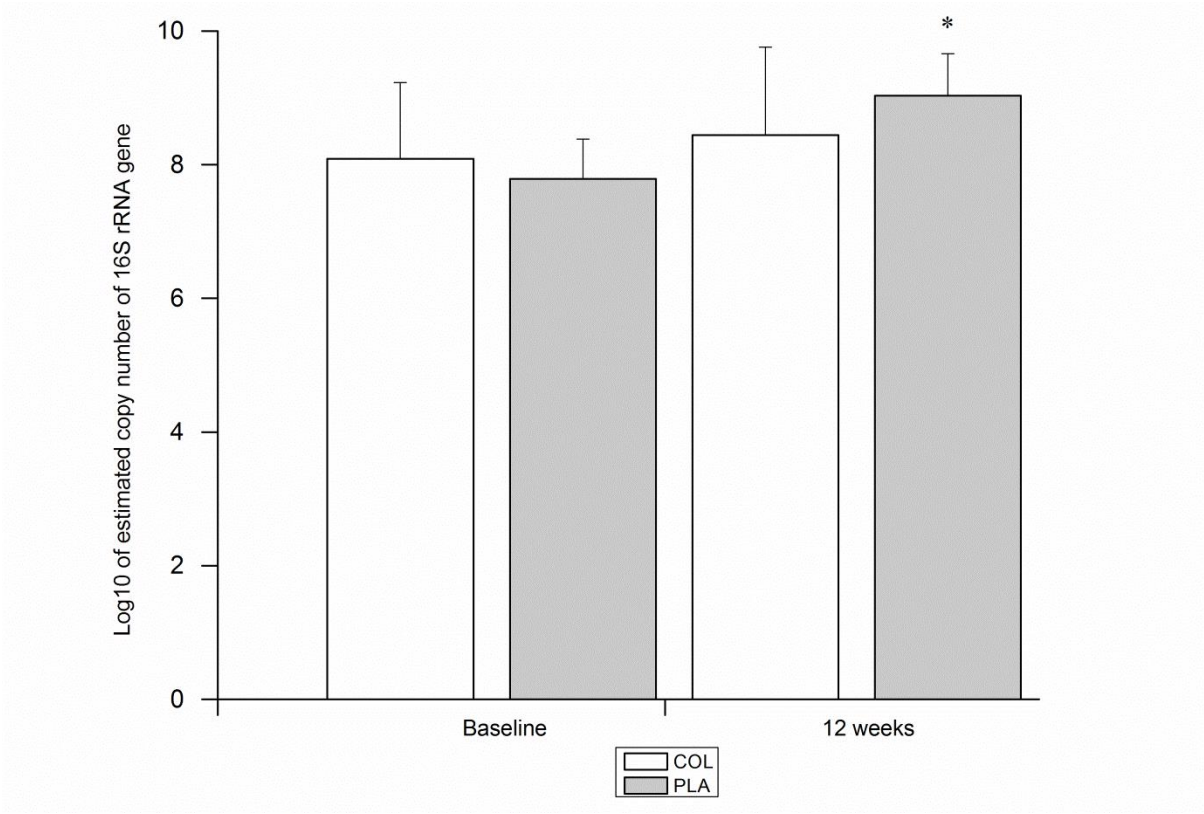

Figure 5.2 Salivary bacterial load in COL/ PLA groups. Standard deviation as error bars. *Significant increase in salivary bacterial load from baseline to 12 weeks in PLA group ($p < 0.001$).

5.3.5 Immune cell counts and neutrophil responses

Results for total and differential leukocyte counts, and stimulated neutrophil oxidative burst, at baseline and 12 weeks following supplementation in COL and PLA groups are shown in Table 5.3.

Table 5.3 Blood leukocytes and neutrophil function in COL and PLA groups.

Immune measure	Baseline	12 weeks	p trial p time p interaction
Total leukocytes (cells $\times 10^9 \cdot L^{-1}$)			0.382
COL	4.74 ± 1.02	4.90 ± 1.01	0.193
PLA	5.20 ± 1.68	5.27 ± 1.63	0.687
Neutrophils (cells $\times 10^9 \cdot L^{-1}$)			0.268
COL	2.31 ± 0.61	2.31 ± 0.59	0.359
PLA	2.58 ± 1.18	2.74 ± 1.28	0.480
Monocytes (cells $\times 10^9 \cdot L^{-1}$)			0.766
COL	0..46 ± 0.11	0.46 ± 0.12	0.567
PLA	0.46 ± 0.13	0.47 ± 0.20	0.431
Total lymphocytes (cells $\times 10^9 \cdot L^{-1}$)			0.970
COL	1.77 ± 0.50	1.91 ± 0.67	0.619
PLA	1.90 ± 0.61	1.79 ± 0.47	0.079
Large immature cells (cells $\times 10^9 \cdot L^{-1}$)			0.305
COL	0.03 ± 0.01	0.03 ± 0.02	0.832
PLA	0.05 ± 0.04	0.04 ± 0.03	0.605
fMLP-stimulated CL per neutrophil ($RLU \cdot s^{-1} \cdot cell^{-1}$)			0.413
COL	43.7 ± 27.4	54.0 ± 26.5	0.025*
PLA	42.8 ± 25.6	44.5 ± 24.9	0.365

Significant difference between baseline and 12 week measures (main effect of time) indicated by * $p < 0.05$.

5.3.6 Serum metabolomics

Sugisawa et al. (2003) have suggested that low molecular weight (< 10 kDa) substances may be responsible for the immunological effects of COL. We therefore used a metabolomics approach to determine whether COL increased the concentration of compounds at the lower end of this range (< 1.4 kDa) with the expectation that any such effects would be due to bioactive metabolites. Thus, metabolite profiles of serum derived using DI-ESI-MS were analysed using multivariate approaches. DFA of serum profiles at baseline and at 12 weeks (Figure 5.3), showed no separation, at either time point, between the COL and PLA groups. However, there was clear separation between the two timepoints. Interrogation of the loading vectors selected by the algorithm to derive DF1 suggested that 13 m/z were calculated to be the major sources of variation. Interrogation of the HMDB Serum Metabolome database allowed the tentative identification of these 13 metabolites (Table 5.4). Within the tentative identifications, there are several possibilities for each m/z but no particular biochemical pathways or immunological metabolite markers appeared to have been targeted.

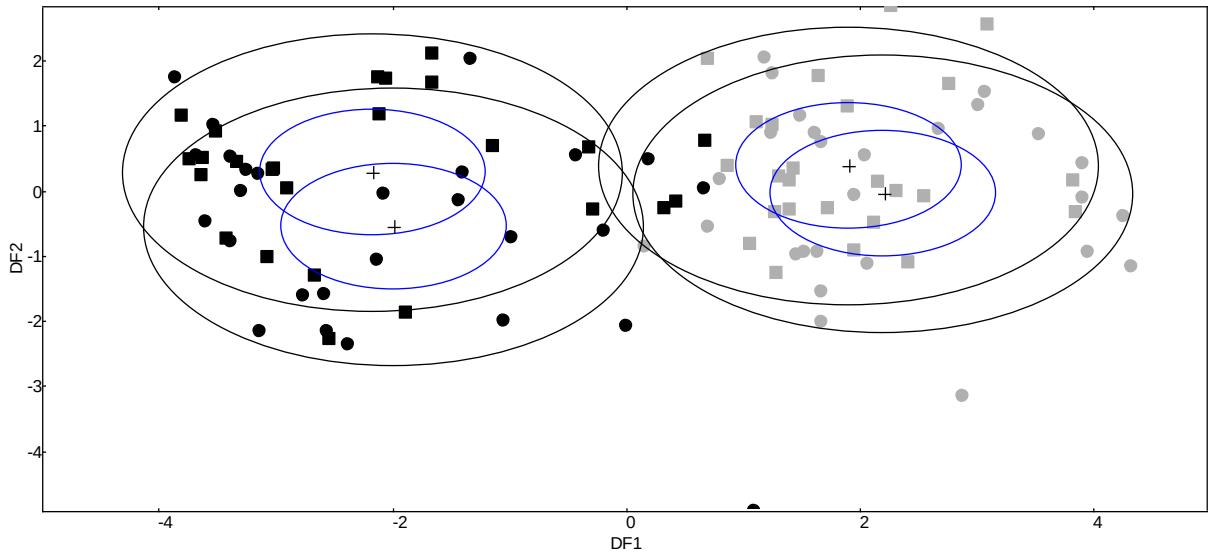

Figure 5.3 Discriminant Function analysis (DFA) plot of metabolomic profiles before and at end of 12 weeks of COL and PLA supplementation. ■ - COL group at baseline; ● - PLA group at baseline; ■ - COL group at 12 weeks; ● - PLA group at 12 weeks. A satisfactory separation was obtained between time points but not groups. Rings on figure display 95% confidence intervals for DFA separation.

Table 5.3 Tentative identifications of metabolites which may be responsible for the separation between the 2 timepoints visible in DF1 of Figure 5.3.

MW	Tentative ID	Change from Baseline	p
108	Cresol	Decrease	0.002
154	Gentisic acid 2-Pyrocatechuic acid Protocatechuic acid Hydroxytyrosol	Decrease	< 0.001
155	L-Histidine	Increase	< 0.001
157	Unknown	Increase	< 0.001
168	Uric acid Homogentisic acid 3-Hydroxymandelic acid 3,4-Dihydroxybenzeneacetic acid Pyridoxamine	Decrease	< 0.001
169	Norepinephrine Pyridoxine 3-Methylhistidine D-Glyceraldehyde 3-phosphate Dihydroxyacetone phosphate	Increase	< 0.001
170	Gallic acid 3,4-Dihydroxyphenylglycol cis-4-Decenoic acid	Increase	< 0.001
172	Glycerol 3-phosphate Glycylproline	Decrease	< 0.001
173	2-Oxoarginine Pyrophosphate	Increase	< 0.001
174	Dehydroascorbic acid Suberic acid N-Acetylornithine L-Arginine	Increase	< 0.001
186	2-Phosphoglyceric acid 3-Phosphoglyceric acid	Decrease	< 0.001
285	Unknown	Increase	< 0.001
325	10-Nitrolinoleic acid	Decrease	< 0.001

For some of the whole number molecular weight bins there are multiple tentative IDs which may or may not contribute to the DF1 loading. Change from the baseline and subsequent ANOVA p values are detailed alongside the tentative IDs.

5.4 Discussion

This study aimed to determine the effects of COL on innate and mucosal markers of immunity and the subsequent incidence of URI in regularly exercising males. Compared to the PLA group, there was a significantly lower number of URI episodes and subsequently less proportion of URI days in the COL group over the 12 weeks. This supports previous evidence in studies of both active and immune-deficient populations (Brinkworth and Buckley, 2003; Crooks et al., 2010; Patel and Rana, 2006). Beneficial effects of COL were most evident during periods of greater prevalence of URI within the study population by reducing the proportion of participants who report URI (hence a greater scope for intervention). As not all participants commenced the study at the same time of year, it is important to recognise that these findings reflect the effects of COL within the timeframe and the incidence of illness in participants of the current study rather than proposing a specific seasonal window where COL affects URI. There was, however, no effect of COL on severity and duration of URI episodes, which supports some previous studies (Brinkworth and Buckley, 2003; Crooks et al., 2006, 2010) but not the beneficial effects reported by Patiroglu and Kondolot (2013).

In contrast to the effect of COL on URI, there was no significant effect of COL on salivary SIgA and AMPs or fMLP-stimulated blood neutrophil oxidative burst. The lack of effect on immune parameters despite differences in URI is in accordance with previous evidence (Crooks et al., 2006; Shing et al., 2007). Although previous studies have found beneficial effects in mucosal protection (salivary SIgA and AMPs) following COL supplementation, either a blunting of the exercise-induced immune dysfunction (Davison and Diment, 2010) or increased resting salivary SIgA (Crooks

et al., 2006), they did not measure URI (Davison and Diment, 2010) or failed to see simultaneous effects of COL on URI (Crooks et al., 2006). This has led to proposals that the effect of COL occurs through a combination of mechanisms (Shing et al., 2007). In the current study we proposed the use of a novel *in vivo* marker (salivary microbiome) that may have greater sensitivity to changes in innate mucosal defense following an intervention. This study shows for the first time that during regular training in the winter months, COL limits the increase in salivary bacterial load that was observed in the PLA group. However, it is currently unclear whether the significant increase in bacterial load in the PLA group played a role in the greater episodes of URI observed in this group or whether the increased bacterial load occurred as result of a greater number of illnesses, or compromised immunity (due to training and/or seasonal effects). Future studies should investigate salivary bacterial load at the taxonomic level with next generation sequencing methods to determine whether changes reflect predisposed interactions with viruses which are the common cause of URI (Bosch et al., 2013).

Evidence from respiratory health research has suggested that bacterial colonisation of tissues contiguous to the oral cavity (e.g. airway) can trigger an increase in the frequency of disease exacerbations (Wedzicha and Donaldson, 2003). Furthermore, the interaction between viruses and bacteria which colonise the upper respiratory tract has been highlighted to affect the risk of illness (Bosch et al., 2013). Of relevance to this study are suggestions that the balance of microbes involved in colonisation can be perturbed when host immunity is compromised (Murphy et al., 2009). It is apparent that the magnitude of change in immunity immediately following each bout of strenuous exercise may have more clinical significance than training-induced alterations in resting immunity (Abbasi et al., 2013; Nieman et al., 1994;

Pedersen and Bruunsgaard, 1995). It is reasonable to suggest that over the course of the 12 weeks the COL group suffered fewer incidences of transient immune perturbations, and/or smaller or shorter disturbance, (in response to training and/or seasonal variation) which may have limited conditions for changes in the salivary microbiome to occur. COL has been shown to prevent exercise-induced decreases in salivary AMPs and improve recovery of the capacity of neutrophils (a source of AMPs) following strenuous exercise (Davison and Diment, 2010). Deficiencies or decreases in AMPs expressed in mucosal secretions have been shown to be related to greater bacterial invasion and/or cases of infection (Bals et al., 1998, 1999; Daele and Zicot, 2000; Smith et al., 1996b; Goldman et al., 1997). It is worthy to note, however, that the present study has only investigated a narrow range of AMPs that are present in mucosal secretions. Therefore, the effects of COL on the resting concentrations of other AMPs such as cathelicidins, which have broad antibactericidal effects (Bals, 2000) remain unclear and cannot be excluded. In addition, there were no apparent effects of COL on *in vitro* microbial culture in this study which supports that the aforementioned effects on bacterial load are not due to components of COL having direct antimicrobial effects in the oral cavity during consumption.

Of particular note was the lack of simultaneous increase in bacterial diversity within the PLA group during the study. This suggests that it was not the acquisition of new bacteria that caused the significant increase in bacterial load in the PLA group but rather amplification of bacteria resident at baseline. The oral cavity is exposed to a constant array of exogenous and endogenous factors, thus measurement via saliva has been recognised to provide a ‘fingerprint’ of the whole oral microbiota (Boutaga et al., 2007; Dewhirst et al., 2010; Fabian et al., 2008; Li et al., 2005;). We propose

that, rather than causing URI *per se*, an increase in bacterial load is indicative of compromised innate immune status, and as such is a relevant marker of *in vivo* (innate) immune status. Furthermore, if COL supplementation does limit increases in salivary bacterial load, it will be important to determine whether this is a general reduction in bacteria, or whether it is biased towards certain bacterial taxonomies.

A further aim of this study was to determine any metabolomic changes as a result of COL supplementation. Metabolomics provides an unbiased biochemical "snap shot" of samples which due to the use of high resolution mass spectrometry-based (as here) or nuclear magnetic resonance-based approaches simultaneously and accurately measure hundreds of metabolites. Exercise immunologists view that high throughput laboratory methods such as metabolomics will provide greater understanding of the mechanisms behind modulations of the immune system with exercise and/or nutrition (Walsh et al., 2011b). Crucially, in this study, we did not observe any changes in serum metabolome linked to COL supplementation, indicating that either no major changes were occurring or that these were occurring below the detection limits of the Mass Spectrometer (< 10^{-12} mol, Sumner et al., 2003). There was, however, a clear separation of metabolome profiles obtained at baseline and at the 12 week timepoint of the study. The timepoint effect could reflect a combination of a seasonal effect and accumulation of training stress. As seasonal and/or exercise effects alone on the immune system were not a primary aim of the study, future studies should investigate whether changes in metabolome are involved in the greater incidence of URI seen in the winter months. Confirmatory identifications of the 13 m/z which appear to be responsible for the majority of the temporal separation were not accomplished as changes in the serum metabolome

were not as a result of COL supplementation and thus fell outside of the scope of this project.

Previous *in vitro* culture studies suggested that low molecular weight (≤ 10 kDa) components of COL (e.g. proteose peptones) rather than larger growth factors or cytokines may be responsible for the effects on human immune function (e.g. neutrophil oxidative burst) (Sugiswa et al., 2003). Given that our metabolomics approach provides data on (< 1.4 kDa) metabolites involved in biological pathways (Holmes et al., 2008), it may have been expected to identify traces of such bioactive metabolites in the circulation following 12 weeks of supplementation. Future studies could additionally examine components between 1.4 - 10 kDA to identify whether the above mentioned components of COL investigated within *in vitro* culture become bioavailable to affect human immune function following periods of supplementation. Such studies will help identify the bioactive components and eradicate discrepancies found between studies of COL that are due to the source/quality of the supplement (Davison, 2013). It is plausible, however, to suggest that in the present study the proposed priming effects may have been localised to immune parameters in the mucosae rather than having systemic effects. Another proposed mechanism behind the effects on URI is the ability of COL to truncate the increase in gut permeability following strenuous exercise (Marchbank et al., 2011). This effect on intestinal integrity may prevent additional stress on the immune system via the translocation of luminal bacteria into systemic circulation. This hypothesis, however, requires further study in relation to episodes of URI.

Although previous studies have shown inconsistencies in isolated immune markers at rest, the present study suggests that the effects of COL are apparent when an

integrated and interactive holistic immune marker is taken (e.g. salivary bacterial load). It should be noted that participants in this study were not limited in their use, either before or during the study, of mouthwash. This could be considered a potential limitation of the study and an important consideration for future studies using the salivary bacterial load as an *in vivo* marker. Given the presence of null findings in the present study compared to previous studies of investigated immune parameters (e.g. salivary SIgA), it is important to acknowledge methodological limitations not yet considered. The present study involved participants who were involved in a range of sports training. Although these were all endurance based activities, previous studies have reported that effects of COL on salivary SIgA may not be universal in all groups of regular exercisers (Crooks et al., 2010). In addition, it is unclear whether the use of participants who had a higher mean weekly training load and hence potentially a greater number of immunodepressive bouts would have produced different findings for these measures. Indeed, when highly trained cyclists completed 5 consecutive days of high-intensity training, COL supplementation was found to prevent the decreases in cytotoxic/suppressor T cells observed at the end and in the recovery period from the training in the PLA group (Shing et al., 2007).

In summary, we have shown for the first time that COL supplementation can limit microbial changes in the oral cavity during the winter months. This may be due to COL acting as a nutritional countermeasure to seasonal effects on salivary microbiome or immune perturbations following acute exercise. The clinical implications of this may be a reduction in the number of URI episodes. Future research should include responses to acute training during the monitoring period to determine the effects on the salivary microbiome and whether COL influences these (and other, e.g. AMPs) responses.

Chapter 6. Study 4 - The effects of bovine colostrum on symptoms of upper respiratory illness in EBV seronegative and seropositive active males

Abstract

Previous infection with EBV has been proposed as a possible risk factor for more frequent illness in those involved in intense training. The aims of this study were to investigate the effects of EBV serostatus on episodes of URI and the total number of URS days across a 12 week period and whether such outcomes would be influenced by COL supplementation. Serum collected from the 53 males of Study 3 (Chapter 5) at baseline and following 12 weeks of COL or PLA supplementation was assayed for IgG antibodies to EBV VCA. There was no difference in the number of URI and URS days or incidence, duration and severity of URI episodes based on EBV serostatus alone ($p < 0.05$). Similar findings were also observed when comparing EBV seropositive and seronegative participants in the PLA group ($p < 0.05$). There was a significant difference in proportion of URI days ($p < 0.05$) and a trend for a lower duration of URI episodes ($p = 0.07$) in seropositive participants of the COL group compared to their seronegative counterparts receiving COL. This study indicated no clear effect of EBV serostatus on URI but within participants who underwent COL supplementation, seropositive males suffered fewer URI days than those who have not previously suffered EBV infection. Further investigations are warranted to determine the role of prior infection with herpesviruses in URI of athletes and how nutritional interventions may mediate the risk.

6.1 Introduction

Exposure of athletes to a greater strain of exercise training (through exceeding individual training thresholds) in combination with other stressors (e.g. inadequate nutrition, psychological) can increase the risk of URI (Foster et al., 1998; Gleeson et al., 2007; Pyne and Gleeson, 1998). For such reasons, athletes tend to report URI either during the high intensity and tapering period prior to competition (e.g. swimming, team sports) or in the period following competition (e.g. long distance running) (Walsh et al., 2011b). Despite such observations, even when significant immune perturbations have been observed in athletes participating in strenuous exercise, investigators have had limited success in identifying such measures that influence alterations in rates of URI (Gleeson et al., 1999; Mackinnon et al., 1993; Nieman, 2000; Nieman et al., 1990b, 2000; 2007).

Previous evidence has shown the benefits of COL supplementation (8-10 weeks) towards reducing the incidence of URI in physically active and in immune deficient populations (Brinkworth and Buckley, 2003; Crooks et al., 2010; Patel and Rana, 2006). This was supported by study 3 (Chapter 5) where following 4 weeks of supplementation there was a lower proportion of days and participants in the COL group who reported URI. Within this study, an increase in bacterial load over the 12 week winter period within the PLA group was limited with COL supplementation. We proposed that, rather than causing URI *per se*, an increase in saliva bacterial load is indicative of compromised innate immune status, and as such is a relevant marker of *in vivo* (innate) immune status.

There is a considerable interest in identifying risk factors which may determine the susceptibility of athletes to URI. Previous infection (i.e. seropositive) with viruses of

the herpes family (e.g. EBV) have been proposed as possible risk factors for more frequent illness in those involved in intense training (He et a., 2013b). EBV typically infects humans early on in life, with up to 90% of the world's population infected by adulthood (Gleeson et al., 2002; Sarid et al., 2001). Gleeson et al. (2002) found a significant correlation between previous EBV infection and URI, while all seronegative swimmers reported no URI during a 1 month period of intensive training. It is worthy to note that this study did include a relatively small sample size and was undertaken over a short window which may not provide sufficient opportunity to provide a valid indicator of an enhanced risk to frequent URI. Indeed, other investigations conducted over a longer period (i.e. 4 months) and included larger sample sizes have found no differences in URI based on EBV serostatus (He et al., 2013b). Others have suggested that prior infection with herpesviruses may provide a symbiotic rather than a deleterious effect on risk of acute illness with other pathogens (Barton et al., 2007; Sandalova et al., 2010). Latent EBV infection may promote immunosurveillance through inducing prolonged elevation of cytokines (e.g. IFNγ) and systemic activation of macrophages (Barton et al., 2007). Furthermore, positive EBV serostatus has been suggested to confer heterologous immunity whereby EBV specific CD8+cells augment the population of T cells which proliferate during acute viral infection (Sandalova et al., 2010).

EBV infects epithelial cells and B cells of the orpharynx where it establishes lifelong persistence in the former (Cruchley et al., 1997; Sixbey et al., 1984). This EBV infected B cell population is tightly-regulated by T^c cells (Rickinson and Moss, 1997). Periods of physical and/or psychological stress have been shown to reduce the effectiveness of EBV specific T^c cells (Glaser, et al., 1999). Such disturbance within certain parameters of the immune system which usually keep the virus dormant can

lead to reactivation of EBV (Mehta et al., 2000a). EBV can replicate continuously or intermittently from the orpharynx and it has been suggested that athletes have greater levels of detectable EBV-DNA compared with non-exercising controls (Faulkner et al., 2000; Hoffmann et al., 2010; Nadal et al., 2002). Therefore, an alternative hypothesis to the proposal that EBV serostatus may affect susceptibility to URI is that the cell damage and inflammation which occurs within the orpharynx during an EBV reactivation may itself induce URS (Cox et al., 2004). This may provide an explanation to the minor, short lasting symptomatology that is commonly found within athletes alongside persistent fatigue as a result of the stress of chronic training (Reid et al., 2004). It may also partly explain the low proportion (30%) of identified pathogens in reported illness (Spence et al., 2007), particularly within EBV seropositive athletes. However, if the appearance of symptoms occur as a result of dysregulated immunity, it is possible that EBV seropositive athletes would benefit from a nutritional countermeasure (e.g. COL) to maintain the integrity of immune parameters which control the latency of EBV.

Further investigation is warranted to determine whether positive EBV serostatus increases the incidence of minor URS or whether it influences susceptibility to episodes of conventional URI. An understanding of the clinical relevance of EBV serostatus may highlight possible prevention and treatment strategies. Numerous studies have investigated the use of nutritional interventions to prevent the risk of URI in athletes but none have explored such effects in relation to latent herpesviruses.

The aims of this study were to investigate the effects of EBV serostatus on the proportion of URI episodes and the total number of URS days (which may include

short-lasting symptomatology related to previous EBV infection) across a 12 week period and whether such outcomes would be influenced by COL supplementation.

6.2 Methods

6.2.1 Participants

Fifty three males, completing at least 3 h of moderate-vigorous endurance exercise a week**,** volunteered to participate in the present investigation.

6.2.2 Supplementation

Participants were randomly allocated into COL or PLA groups with stratification by age and type of exercise training only. Participant characteristics of each group (COL = 25, PLA = 28) are detailed in a companion study (Study 3). In a double blind manner, participants were asked to consume 20 $g \cdot day^{-1}$ (10 g prior to morning and evening meal) of COL (Neovite UK, London) or an isoenergetic/isomacronutrient PLA (as used in study 3) for 12 weeks.

6.2.3 EBV serology

Serum collected from participants at baseline and following 12 weeks of supplementation was assayed for IgG antibodies to EBV viral capsid antigen (VCA) using a commercially available ELISA kit (DRG Diagnostics, Marburg, Germany) according to the manufacturer's instructions. EBV VCA IgG has been recognised to diagnose both previous and current EBV infection (Hess et al., 2004).

6.2.4 Monitoring of upper respiratory symptoms

Participants completed a health questionnaire (Gleeson et al., 2011, 2012a) on a daily basis to indicate any URI (detailed in section 2.8). Participants were also asked to report how their training was affected by the URI (1 – training maintained, 2-training reduced, 3 – training discontinued). Separately to the monitoring of URI (episodes with a total symptom score ≥12), any reported URS, regardless of duration and/or severity, were included in the total URS days for all groups. This was deemed suitable in the present investigation as EBV reactivation has been shown to fluctuate on a daily basis (Gleeson et al., 2002; Yamauchi et al., 2011) and thus would provide a better indication of whether EBV serostatus affects reporting of symptoms.

6.2.5 Statistical analysis

The analysis below was completed in accordance with the methods of He et al (2013b) to determine the influence of EBV serostatus on URI and/or total URS days in the study cohort but with the additional attempt to identify whether any effects would be similar in PLA or COL groups. Data for the proportion of reported URI days and proportion of participants who suffered URI over the 12 weeks between participants who were EBV^+ and EBV^- were assessed by chi-squared test. This was replicated for 2 other comparisons: COL^+ vs. COL^-; PLA^+ vs. PLA^-. Chi-squared analysis was also used to assess proportion of participants who reported use of medication or a negative effect on training load (reduced or discontinued) during URI episodes and proportion of total URS days within the previously mentioned comparative groups. Differences between the comparative groups for mean duration of URI, mean severity of URI and mean weekly training loads were performed with an independent t-test.

6.3 Results

6.3.1 EBV serostatus

Analysis of EBV VCA IgG levels demonstrated that 75% of the PLA group (n = 21) and 60% of the COL group (n = 15) were seropositive (EBV^+). All participants were EBV^+ or seronegative (EBV^-) at both baseline and at 12 weeks, indicating no incidence of acute EBV infection during the study period.

6.3.2 Baseline characteristics and training loads

Baseline characteristics of the subgroups from all 53 participants are provided in table 6.1.

Table 6.1 Baseline characteristics.

Group	N	Age	Body mass	Height
EBV^+	36	32.8 ± 14.1	77.4 ± 8.9	179.1 ± 6.7
EBV^-	17	27.2 ± 11.3	72.3 ± 7.7	179.0 ± 6.3
COL^+	15	33.5 ± 15.1	80.0 ± 9.3	180.3 ± 6.5
COL^-	10	25.9 ± 10.8	72.9 ± 6.3	179.3 ± 6.5
PLA^+	21	32.2 ± 13.6	75.5 ± 8.3	178.3 ± 6.9
PLA^-	7	29.1 ± 12.7	71.4 ± 9.9	178.5 ± 6.5

Mean weekly training volume at moderate-vigorous intensity for the 53 participants across the 12 weeks was 50.5 ± 28.9 MET-h·week^{-1}. There were no significant differences between groups in weekly training loads ($p < 0.05$) (Table 6.2).

Table 6.2 Weekly training loads of moderate-vigorous intensity.

Weekly training load (MET-h·week^{-1})		p
EBV^+	EBV^-	
50.9 ± 27.7	49.7 ± 32.2	0.885
COL^+	COL-	
53.0 ± 24.3	53.2 ± 38.0	0.911
PLA^+	PLA^-	
49.4 ± 30.4	44.7 ± 23.3	0.710

P values are provided for the independent t-tests performed on comparative groups.

6.3.3 Upper respiratory illness and total daily symptoms

There was no significant difference in proportion of URI days (p = 0.457) or total URS days (p = 0.454) between EBV^+ (7% and 11% respectively) and EBV^- groups (7% and 11% respectively). There was also no differences between groups for proportion of participants to report URI (EBV^+: 53%; EBV^-: 53%; p = 0.612), use of medication (EBV^+: 74%; EBV^-: 56%; p = 0.337) and/or negative effect on training during URI episodes (EBV^+:47%: ;EBV^-: 44%; p = 0.885) or the mean number (EBV^+:0.6 ± 0.7; EBV^-: 0.7 ± 0.7; p = 0.968), duration (EBV^+:9.3 ± 5.7 days; EBV^-: 9.4 ± 4.0 days; p = 0.976) and severity (EBV^+:41.4 ± 27.5; EBV^-: 35.9 ± 26.8; p = 0.589) of URI episodes.

There was a significant difference in proportion of URI days (p = 0.001) between COL^+ (3%) and COL^- (6%). There was, however, no differences between groups for total URS days (COL^+: 8%; COL^-: 9%; p = 0.138), proportion of participants to report URI (COL^+: 40%; COL^-: 40%; p = 1.000), use of medication (COL^+: 50%; COL^-:

50%; p = 1.000) and/or negative effect on training during URI episodes URI (COL^+: 50%; COL^-: 75%; p = 0.429) or the mean number (COL^+: 0.5 ± 0.6; COL^-: 0.5 ± 0.7; p = 0.904), duration (COL^+: 6.0 ± 2.1 days; COL^-: 10.6 ± 5.7 days; p = 0.074) and severity (COL^+: 28.4 ± 17.7; COL^-: 45.0 ± 36.3; p = 0.313) of URI episodes.

There was no significant difference in proportion of URI days (p = 0.426) or total URS days (p = 0.209) between PLA^+ (13% and 10%, respectively) and PLA^- (13% and 9%, respectively). There was also no differences between groups for proportion of participants to report URI (PLA^+: 62%; PLA^-: 71%; p = 0.649), use of medication (PLA^+: 85%; PLA^-: 60%; p = 0.261) and/or negative effect on training during URI episodes (PLA^+: 46%; PLA^-: 17%; p = 0.216) or the mean number (PLA^+:0.8 ± 0.7; PLA^-: 0.9 ± 0.7; p = 0.976), duration (PLA^+:10.8 ± 6.2 days; PLA^-: 8.3 ± 1.8 days; p = 0.174) and severity (PLA^+:47.1 ± 29.5; PLA^-: 28.3 ± 15.0; p = 0.157) of URI episodes.

6.3.4 Total and differential leukocyte counts

Analysis of leukocyte changes from baseline to 12 weeks indicated a significant difference between EBV^+ and EBV^- groups across time in monocyte counts (2 way ANOVA group × time interaction, p = 0.037). Post hoc analysis could only reveal a trend for a decrease across time in EBV^- (p = 0.052). There were no main effects of time or group in monocyte counts (p > 0.05). Results for total leukocytes, neutrophils, total lymphocytes, neutrophil:lymphocyte, revealed no main effects of time, group, or group × time interaction (p > 0.05) (Table 6.3).

Table 6.3 Total and differential leukocyte counts at baseline and 12 weeks timepoints in EBV^+ and EBV^- participants.

Cell count, $10^9 \cdot L^{-1}$	Baseline	12 weeks
Total leukocytes		
EBV+	5.02 ± 1.49	5.24 ± 1.46
EBV-	4.91 ± 1.28	4.79 ± 1.15
Neutrophils		
EBV+	2.47 ± 1.05	2.64 ± 1.14
EBV-	2.42 ± 0.75	2.31 ± 0.73
Monocytes		
EBV+	0.45 ± 0.12	0.48 ± 0.17
EBV-	0.47 ± 0.12	0.44 ± 0.14
Total lymphocytes		
EBV+	1.86 ± 0.54	1.86 ± 0.58
EBV-	1.79 ± 0.60	1.82 ± 0.56
Neutrophil:lymphocyte		
EBV+	1.40 ± 0.62	1.51 ± 0.69
EBV-	1.44 ± 0.53	1.36 ± 0.62

Analysis of total and differential leukocyte counts between COL^+ and COL^- at baseline and 12 weeks timepoints revealed no main effects of group, time or group × time interaction ($p > 0.05$) (Table 6.4). There was a significant difference in total leukocyte changes across time between PLA^+ and PLA^- groups (2 way ANOVA group × time interaction, $p = 0.046$) (Table 6.4). Post hoc analysis of timepoints could not reveal significant differences within groups. All other leukocytes counts revealed no main effects ($p > 0.05$), although a trend did emerge for group × time interaction in monocyte counts ($p = 0.068$) (Table 6.4).

Table 6.4 Immune cell counts at baseline and 12 weeks following COL or PLA supplementation in EBV^+ and EBV^- participants.

	COL		PLA	
Cell count, $10^9 \cdot L^{-1}$	Baseline	12 weeks	Baseline	12 weeks
Total leukocytes				
EBV^+	4.66 ± 0.81	4.82 ± 0.92	5.28 ± 1.80	5.53 ± 1.71
EBV^-	4.87 ± 1.31	5.02 ± 1.18	4.96 ± 1.35	4.47 ± 1.12
Neutrophils				
EBV^+	2.21 ± 0.39	2.24 ± 0.34	2.66 ± 1.32	2.93 ± 1.40
EBV^-	2.46 ± 0.85	2.41 ± 0.85	2.37 ± 0.65	2.16 ± 0.55
Monocytes				
EBV^+	0.45 ± 0.15	0.46 ± 0.12	0.46 ± 0.14	0.49 ± 0.22
EBV^-	0.50 ± 0.23	0.54 ± 0.12	0.46 ± 0.12	0.40 ± 0.10
Total lymphocytes				
EBV^+	1.79 ± 0.43	1.90 ± 0.73	1.90 ± 0.62	1.84 ± 0.47
EBV^-	1.73 ± 0.60	1.92 ± 0.61	1.88 ± 0.64	1.67 ± 0.48
Neutrophil:lymphocyte				
EBV^+	1.27 ± 0.28	1.28 ± 0.31	1.49 ± 0.77	1.67 ± 0.84
EBV^-	1.50 ± 0.56	1.37 ± 0.78	1.36 ± 0.51	1.34 ± 0.30

6.4 Discussion

The aims of this study were to determine the influence of prior EBV infection on susceptibility to episodes of URI or short-lasting URS and identify whether the likelihood of such outcomes would be influenced by COL supplementation. The present study suggests that EBV serostatus alone does not affect the number of URI/URS days or the duration and severity of URI episodes. This is in support of the evidence from the recent study by He et al. (2013b) but not the findings of Gleeson et al. (2002). In line with He et al. (2013b) the present study involved monitoring a larger cohort of participants from a range of endurance sports compared to the small numbers of elite swimmers in Gleeson et al. (2002). More investigations are warranted to clarify whether susceptibility to URI due to EBV serostatus may be specific to certain athletes/sport settings (e.g. swimmers) undergoing intensified training.

It is worthy to note there was a modest (3%) difference in the proportion of URI days between COL^{+} and COL^{-}. The reasons for this and the biological significance are still unclear but may purely reflect certain pathogens involved in the illnesses of each group as there were similar trends for URI duration and no effect on number of URI episodes. Prior EBV infection has been shown to alter populations of CD8+ T cells which may produce antiviral cytokines. He et al. (2013b) observed fewer URI episodes and days and greater total lymphocyte count at rest in athletes who had prior CMV and EBV infection compared with athletes who were seronegative for both herpes viruses. This was contrary to their original hypothesis, but as there were also differences in URI days between positive CMV and negative CMV athletes it appears

that previous CMV infection may be an important factor in promoting immunosurveillance.

As only EBV serostatus was assessed in the present study, the role of CMV in differences between COL subgroups can not to be dismissed, particularly as being EBV^+ is more common with positive CMV serostatus (Bigley et al., 2012). This would not explain why there were no such differences in URI between PLA^+ and PLA^- of this study. Furthermore, in the present study there were no apparent differences in leukocyte counts between COL^+ and COL^- to be suggesting any higher level of immunosurveillance. CMV serostatus has been shown to amplify memory CD8+ T cells to acute exercise (Turner et al., 2010). In contrast, others have reported blunting of NK cell mobilisation following exercise in CMV positive participants (Bigley et al., 2012), or found no effect of CMV serostatus on responses of granulocytes and monocytes (LaVoy et al., 2013). At present, the effect of EBV serostatus on response to acute exercise is understudied; further research is required to clarify the role of prior infection with CMV/EBV on acute response to exercise and whether use of a nutritional intervention (e.g. COL) can have additive effects on any immunosurveillance.

Although EBV serostatus was not assessed in Yamauchi et al. (2011), salivary expression of EBV-DNA tended to occur during reporting of URS (cough, sore throat) in rugby players involved in an intensive period of training. The present study design did not allow for the exploration of any direct role of EBV reactivation in causing URS. Notwithstanding, the lack of differences in total URS days between groups based on EBV serostatus may provide indirect evidence to suggests that being EBV^+ does not increase the incidence of URS. The appearance of EBV in

saliva may be random, an epiphenomenon to URS incidence, with no clinical significance *per se* on athlete's health (Gleeson et al., 2002). However, the lack of investigation of EBV expression during URS in the present study must be considered a limitation as determining the relationship between total URS with EBV serostatus alone may be masked by other non-infectious inflammatory stimuli (Bermon, 2007).

It is unclear whether the use of participants who had a higher mean weekly training load and/or of similar status to previous studies (e.g. elite, Gleeson et al., 2002), hence with the potential for a greater risk of EBV reactivation, would have produced different findings in total URS. There is reasonable evidence to suggest that EBV reactivation during intensified periods of training may be a marker of compromised mucosal and/or cellular immunity rather than contributing towards a risk for URI (Gleeson et al., 2002: Mehta et al., 2000a). Such perturbations in the T^c population may explain why decrements in mucosal protection (i.e. secretory IgA) have also been found in conjunction with increased EBV expression (Gleeson et al., 2002; Yamauchi et al., 2011). Further evidence to support proposals of salivary EBV expression as an *in vivo* marker of immunodepression only are findings of administration of antiviral (specific to herpes viruses) agents reducing detectable EBV but yet not influencing the incidence of URI in endurance runners (Cox et al., 2004).

The importance of detecting novel indicators of immune dysfunction and risk factors for illness is highlighted in the present study by the negative effect on training volume in ~ 46% (both groups combined) of URI episodes. Regardless of any role in symptomatology, as increased EBV expression has been shown to coincide with immune perturbations during physiological and/or psychological stressors (Mehta et

al., 2000a, 2000b), it may provide a useful *in vivo* marker of the effects of training on the immune system of athletes. Although EBV seroprevalence was somewhat lower in the present cohort (~ 68%) compared to previous reports in the general population (80-90%), the high proportion of seropositve in the adult population reiterates the need for further research on this latent virus.

In summary, the current study indicated that there was no clear effect of EBV serostatus on URI but within participants who underwent COL supplementation, EBV^+ suffered fewer URI days than their EBV^- counterparts. This study contributes to on-going research exploring the role of latent viruses towards the risk of URI. Further investigations are warranted to determine the role of prior EBV infection towards responses to acute endurance exercise and/or duration of URI and whether nutritional interventions influence the significance of any changes.

Chapter 7. Study 5 - The effects of bovine colostrum supplementation on *in vivo* T-cell-mediated responses within a contact hypersensitivity model following prolonged exercise

Abstract

Prolonged exercise significantly reduces both the induction and elicitation of *in vivo* cell-mediated immune responses. The aim of this study was to investigate the effects of COL supplementation on the *in vivo* cell-mediated responses to a topical sensitiser (Diphenylcyclopropenone, DPCP) following prolonged exercise. In a double-blind design, 28 male participants were randomly assigned to either a COL (20 g a day) (n = 14, age: 24 ± 5 years, BM: 75.6 ± 9.5 kg, height: 181 ± 6 cm, $\dot{V}O_2$ max: 57.1 ± 5.2 mL·kg^{-1}·min^{-1}) or PLA (n =14, age: 25 ± 6 years, BM: 74.1 ± 8.6 kg, height 179 ± 8 cm, $\dot{V}O_2$max: 58.4 ± 8.2 mL·kg^{-1}·min^{-1}) group for 87 days following stratification for age and aerobic fitness only. Exactly 28 days into supplementation, participants took part in 2 h of running at 60% of $\dot{V}O_2$max. Within 20 min of exercise completion, all participants were sensitised to DPCP using a single patch applied to the mid-lower back for 48 h. Following the induction of immune-specific memory (sensitisation), participants reported to the laboratory 28 days later for a dose series of DPCP patches to be applied in a randomly allocated order to the volar aspect of their right upper arm for 6 h. Participants returned to the laboratory 24 h and 48 h following the application of patches for skin responses (oedema) to be measured at each DPCP patch site using modified skinfold callipers. There was no difference in total oedema responses (sum of all skinfold sites) between COL and PLA ($p > 0.05$). Analysis of the dose response curves allowed for the minimum dose (threshold) for a positive response in each group to be determined (i.e. sensitivity). The minimum dose for PLA was 2.0 and 2.1 fold greater than COL at 24 h and 48 h respectively

(i.e. greater sensitivity in COL). There was a greater response in COL at 24 h for the lowest DPCP dose ($p < 0.05$), but not at 48 h or with other doses at either timepoint ($p > 0.05$). There were no apparent effects of COL supplementation on the magnitude of cutaneous immune responses (summed skinfold responses) at recall (4 weeks following initial sensitisation to DPCP). The study does, however, suggest that COL enhances sensitivity of the recall of antigen-specific memory. This may support previous evidence from our laboratory where COL has been shown to act as a nutritional countermeasure to prolonged exercise and decrease susceptibility to illness.

7.1 Introduction

Numerous studies have shown significant decreases in circulating and mucosal immune function in individuals undergoing heavy physical exertion (Nieman, 2007). The majority of these studies have investigated this via animal studies or *in vitro* measures (Walsh et al., 2011b). The relevance of animal studies to human immune function comes under constant scrutiny while clinical relevance of investigations using *in vitro* measures have been questioned, particularly if cells have been isolated from their normal environment (Vumanovic-Stejic et al., 2006). The importance of *in vivo* measures of immunity to determine the clinical relevance of an intervention have been recognised (Albers et al., 2005). The few human studies which have used these measures have investigated the *in vivo* response to novel or previously encountered antigens (Harper-Smith et al., 2011).

Bruunsgaard et al. (1997) found that the DTH skin response to an intra-dermal injection of seven previously encountered antigens (Mérieux CMI Multitest®) was significantly decreased in endurance athletes after prolonged exercise in comparison with rested controls. This technique, however, is limited by the investigation of pre-existing immunological memory (elicitation) and therefore provides no indication of the effects of exercise on the primary (induction) T-cell-mediated response to a novel antigen (Harper-Smith et al., 2011).

Topical skin exposure to novel chemicals (contact sensitisation) such as DPCP allows for the effects of systemic stressors on the induction and elicitation phases of *in vivo* T-cell-mediated immune response to be quantified by oedema and erythema (Albers et al., 2005; Friedmann, 2007; Palmer and Friedmann, 2004). By use of this model of experimental contact sensitisation, Harper-Smith et al. (2011)

demonstrated that participation in a single bout of prolonged (2 h) moderate exercise compared to rest significantly reduced both the induction as well as the elicitation of *in vivo* cell-mediated immunity. Interestingly, in this study the impairment in cell-mediated immunity by a prolonged exercise bout was greater in the induction phase (53%) compared to the elicitation phase (19%). Nevertheless, this supports evidence from other investigations, to date, which indicates that prolonged exercise reduces *in vivo* cell mediated immune response in humans (Bruunsgaard et al., 1997; Dhabhar and Viswanathan, 2005; Edwards et al., 2006; Walsh et al., 2011b).

Several nutritional interventions have been proposed as countermeasures to exercise-induced immune dysfunction. Despite recognition of DTH responses as sensitive *in vivo* indicators of the ability of nutritional interventions to influence cell-mediated immune responses (Bogden et al., 1994; Fuller et al. 1992; Herraiz et al., 1998; Meydani et al., 1990; Pallast et al., 1999), none have explored the use of previously mentioned human *in vivo* model to investigate the effects of nutritional countermeasures to exercise-induced immune dysfunction. Animal and *in vitro* culture studies demonstrate that COL has a mediating effect on cell-mediated immunity by influencing the production of cytokines (Biswas et al., 2007; Shing et al. 2009b). Although some have demonstrated alterations in the balance of T^h1/T^h2 cytokines towards a profile which promotes cell-mediated immunity (Shing et al., 2009b), this may depend on the nature of the stimulus. Biswas et al. (2007) indicated that COL enhanced IFNγ production by PBMC under weak antigenic stimulation but inhibited IFNγ with strong antigenic stimulation (Biswas et al., 2007).

Despite findings of a reduced incidence of URI with COL supplementation in study 3, any effect on immune parameters (at rest) appeared to have been localised to the

oral cavity. Previous investigations (Study 1, 2; Davison and Diment, 2010) suggest that COL can have a beneficial effect on fMLP-stimulated oxidative burst and bacterial-stimulated elastase release of neutrophils within a model of exercise-induced immune dysfunction. It may be hypothesised that the immunomodulatory and immunoprotective effects of COL are more apparent in the immediate period following acute, heavy exertion. Although Albers et al. (2005) suggested that the measure of neutrophil oxidative burst does provide sufficiently reliable and sensitive investigation of a human intervention, it is difficult to conclude with any degree of certainty that increases in one component of immune function is responsible for the alteration observed in an individual's susceptibility to URI with COL supplementation (Brinkworth and Buckley, 2003; Crooks et al., 2010; Patel and Rana; 2006; Patiroglu and Kondolot, 2013; Study 3). To date, most exercise studies (Study 1, 2; Davison and Diment, 2010) have focused on the influence of COL supplementation on innate immunity. The effects of COL on the *in vivo* response to a novel antigen involving a multi-cellular response remain unclear. Therefore, the aim of this study was to investigate the effects of COL supplementation on the induction of a cell-mediated response to DPCP following prolonged exercise.

7.2 Methods

7.2.1 Participants

Twenty eight, healthy, recreational active males, (age 24 ± 5 years; BM 74.9 ± 8.9 kg; height 180 ± 7 cm; $\dot{V}O_2$max 57.8 ± 6.8 mL· kg^{-1}·min^{-1}) volunteered to participate in the present investigation.

7.2.2 Supplementation

In a cross sectional design, 28 male participants were randomly assigned to either the COL group (n = 14, age: 24 ± 5 years, height: 181 ± 6 cm, BM: 75.6 ± 9.5 kg; $\dot{V}O_2$max 57.1 ± 5.2 mL· kg^{-1}· min^{-1}) or a PLA group (n =14, age: 25 ± 6 years, height 179 ± 8 cm, body mass 74.1 ± 8.6 kg; $\dot{V}O_2$max 58.4 ± 8.2 mL·kg^{-1}·min^{-1}) following stratification for age and aerobic fitness only. In a double blind manner, participants were asked to consume 20 g a day (split into a morning and evening 10 g dose) of COL or an isoenergetic/isomacronutrient PLA for ~ 8 weeks (87 days).

7.2.3 Preliminary visits

To control for aerobic fitness prior to group allocation (above), each participant performed a continuous incremental test (1 km·h^{-1} ramp rate every 1 min following 3 min at a speed of 7 km·h^{-1}) to volitional exhaustion on a treadmill with a 1% grade (PPS 55med, Woodway GmbH, Weil am Rhein, Germany). Throughout the duration of the incremental test, expired gas was analysed by the use of an online breath-by-breath gas analysis system (Jaeger Oxycon Pro, Hoechberg, Germany). Upon volitional exhaustion, participants supported their weight with their hands and moved their feet to the sides of the treadmill. Strong verbal encouragement was provided in the later stages of the test to encourage maximal effort. For each participant, $\dot{V}O_2$ max was determined by the highest 30 s average during the test.

At least 48 h following the incremental test and after an overnight fast (from midnight), participants reported to the laboratory at 10:30 for a blood sample (BAS) prior to commencing the above supplementation. Fourteen days into the supplementation period, $\dot{V}O_2$max of participants was determined as detailed

previously. The objective of this second incremental test was to estimate a speed equivalent to 60% $\dot{V}O_2$max for each participant. Seven days later, participants performed a familiarisation trial consisting of a 1 h run at 60% $\dot{V}O_2$max. The main objectives of this visit were to habituate participants with procedures of the main experimental trial (on day 28) and to verify that the speed set from the incremental test corresponded to 60% $\dot{V}O_2$max.

7.2.4 Experimental trials

For the 24 h prior to the main experimental trial, participants were provided a standardised diet of 60% (energy from) carbohydrate: ~5.4 g·kg^{-1} BM; 25% fat: ~1.0 g·kg^{-1} BM; 15% protein: ~1.3 g·kg^{-1} BM and water: 35 mL·kg^{-1} BM (Todorovic and Micklewright, 2004). This diet matched the estimated daily energy expenditure requirements for each participant which was calculated by the equation of Harris and Benedict (1918) multiplied by a physical activity factor of 1.5 (note: relative rest was required on this day). In addition to controlling for the quantity of food, all participants were given the same types of food. This diet did not include any caffeine or alcohol and participants were also asked to avoid any exercise during this period. On day of the main trial, participants reported to the laboratory at 07:30 for a standardised breakfast (total energy: 7.5 kcal·kg^{-1} BM, carbohydrate: ~1 g·kg^{-1} BM [60%], fat: ~ 0.2 g·kg^{-1} BM [25%] and protein: ~0.2 g·kg^{-1} BM [15%]).

Subsequently, all participants had a ~ 3 h period in the laboratory where they completed light activity (e.g. reading, writing, watching TV). Participants were provided a bolus of water equivalent to 5 mL·kg^{-1} BM during this period. A blood sample (PRE) was collected (10:45) prior to participants commencing (11:00) a 2 h running bout at 60% $\dot{V}O_2$max. All participants were permitted diluted cordial (four

volumes of water to 1 volume of sugar-free cordial at 2 mL per kg of BM) every 15 min during the exercise but not at end of the exercise. Expired gas was analysed during the 30th, 60th and 90th minute of exercise (Jaeger Oxycon Pro, Hoechberg, Germany). HR and RPE were monitored and recorded every 15 min during the protocol using a telemetric device (Polar S610, Polar Electro Oy, Kempele, Finland) and the Borg rating scale (Borg, 1982) respectively. A venous blood sample was collected immediately post-exercise (POST). Participants showered and returned to the laboratory within 20 min of exercise completion.

At the same time of day (13:20), participants were sensitised to DPCP using a single patch (comprising of a12 mm aluminium Finn chamber, on Scanpor hypoallergenic tape, containing a 10 mm paper disc) applied to the mid-lower back. The patch had 22.8 µL of 0.125% DPCP in acetone applied (30 µg·cm^{-2} DPCP). This was allowed to dry before application. Following application, the patch remained in place for exactly 48 h. Participants were instructed to avoid alcohol and exercise during this period. Prior to leaving the laboratory on the day of the experimental trial, participants were given a standardised meal (carbohydrate: 0.62 g·kg^{-1} BM (50%), fat: 0.2 g·kg^{-1} BM (34%) and protein: 0.2 g·kg^{-1} BM (16%)). Upon removal of the DPCP patch (48 h later), sensitisation to the novel antigen was assessed (i.e. red mark present at patch site). As used previously (Harper-Smith et al., 2011), participants were contacted 14 days later by telephone to confirm sensitisation.

Exactly 28 days following the induction of immune-specific memory (sensitisation), participants reported to the laboratory for a dose series of DPCP patches to be applied to the volar aspect of their right upper arm (10 µL of DPCP: 0.0048%, 1.239 µg·cm^{-2}; 0.0076%, 1.982 µg·cm^{-2}; 0.0122%, 3.172 µg·cm^{-2}; 0.01953%, 5.075 µg·cm^{-}

^{2}; 0.03125%, 8.120 µg·cm^{-2} and 10 µL of 100% acetone control patch [i.e. 0% DPCP] for background subtraction) at the same time that the initial sensitisation patch was placed on the mid/lower back (13:20). Each individual patch comprised of an 8 mm aluminium Finn chamber mounted on Scanpor hypoallergenic tape containing a 7 mm filter paper disc. To minimise anatomical variability these patches were applied in a randomly allocated order. Following 6 h of application, participants were asked to return to the laboratory for removal of the patches. Participants returned to the laboratory 24 and 48 h following the application of patches for skin responses (oedema) to be measured. Mean skin fold thickness (oedema) was determined from triplicate measurements (to the nearest 0.1 mm) at each DPCP patch site using modified skin fold callipers (Baty, West Sussex, UK). Mean background values were determined from triplicate measurements at the control (0%) patch site, for oedema. All measurements were made by the same investigator to minimise variability in the measurements. Increases in thickness were determined by subtracting the control site value from each DPCP site value. Summed increases in skinfold thickness were determined by adding values over all doses. In the 24 h prior to application of these patches and until measurement of skin responses were complete, participants were requested to avoid any exercise and alcohol.

7.3 Results

7.3.1 Physiological responses

There was no significant difference in $\dot{V}O_2$ (p = 0.692; p = 0.578) between the COL (2656 ± 262 ml·min^{-1}; 61.4 ± 2.3% $\dot{V}O_2$ max) and PLA trials (2702 ± 322 ml·min^{-1}; 62.0.0 ± 3.5% $\dot{V}O_2$max). Mean HR of 149 ± 14 bpm and 149 ± 11 bpm during COL

and PLA trials respectively, was not significantly different (p = 0.968). There was no significant difference in RPE (p = 0.994) between the COL (12.7 ± 1.0) and PLA (12.7 ± 1.2) trials. There was no significant time (p = 0.571), trial (p = 0.092) or interaction effect (p = 0.838) on blood glucose (Table 7.1). There was a significant time effect for blood lactate (p < 0.001) with a significant increase from PRE (p < 0.001) to POST but there was no trial (p = 0.338) or interaction effect (p = 0.105) (Table 7.1).

Table 7.1 Blood glucose and lactate responses in the main experimental trial.

Measure, mmol·L^{-1}	PRE	POST	p values trial time interaction
Blood glucose			0.092
COL	4.13 ± 0.40	4.21 ± 0.50	0.571
PLA	3.91 ± 0.39	3.95 ± 0.49	0.838
Blood lactate			0.338
COL	0.59 ± 0.14	0.71 ± 0.19	< 0.001*
PLA	0.57 ± 0.13	0.84 ± 0.22	0.105

*Significant main effect of time (p < 0.001).

7.3.2 Immune cell counts

No significant trial or interaction effects were evident for total or differential leukocyte counts (Table 7.2). A main effect of time was observed in all leukocyte counts (Table 7.2). There was a significant increase in total leukocytes, neutrophils, monocytes, lymphocytes, neutrophil:lymphocyte ratio and large immature cells from timepoints pre-exercise (BAS and PRE) to POST (p < 0.05). There was no significant change in leukocytes from BAS to PRE except for an increase in neutrophil count (p = 0.026).

Table 7.2 Immune cell counts prior to and following prolonged running.

Cell count, $10^9 \cdot L^{-1}$	BAS	PRE	POST	p values trial time interaction
Total leukocytes				0.537
COL	5.49 ± 1.75	5.65 ± 1.33	11.10 ± 3.96	< 0.001*
PLA	4.80 ± 1.23	5.18 ± 0.94	11.76 ± 5.71	0.435
Neutrophils				0.533
COL	2.98 ± 1.69	3.21 ± 1.27	8.07 ± 4.33	< 0.001*
PLA	2.36 ± 0.81	2.85 ± 0.78	8.24 ± 5.09	0.484
Monocytes				0.622
COL	0.55 ± 0.17	0.53 ± 0.12	0.83 ± 0.32	< 0.001*
PLA	0.47 ± 0.12	0.51 ± 0.13	0.89 ± 0.40	0.275
Total lymphocytes				0.653
COL	1.72 ± 0.35	1.59 ± 0.35	2.24 ± 0.67	0.001*
PLA	1.77 ± 0.68	1.62 ± 0.58	2.36 ± 0.57	0.866
Neutrophil:lymphocyte				0.434
COL	1.91 ± 1.69	2.13 ± 1.01	3.71± 1.66	< 0.001*
PLA	1.37 ± 0.40	1.82 ± 0.54	3.51 ± 1.93	0.640
Large immature cells				0.771
COL	0.05 ± 0.04	0.05 ± 0.03	0.15 ± 0.10	< 0.001*
PLA	0.04 ± 0.02	0.04 ± 0.01	0.20 ± 0.17	0.485

*Significant main effect of time ($p < 0.001$).

7.3.3 Contact Hypersensitivity responses

Two way ANOVA revealed there was a significant increase in summed skinfold responses from 24 h to 48 h (< 0.001) (Figure 7.1). However, there was no effect of trial (p = 0.519) or trial × time interaction (p = 0.943). Data for individual doses could not be normalised for 2-way ANOVA analysis. As the main purpose of the study was to assess the potential effects of COL on *in vivo* responses to a range of DPCP doses and due to apparent differences in sensitivity between groups (see below), individual doses were investigated by non-parametric (Mann Whitney-U) tests (Figure 7.2). At 24 h for the lowest dose of DPCP, there was a significantly greater response in COL compared to PLA (p = 0.039). There were no other differences between the COL and PLA group at 24 h or 48 h for any of the other doses of DPCP (p > 0.05).

In accordance with Harper-Smith et al. (2011), further analysis of the dose response curves (Figure 7.2) would allow the minimum dose (threshold, x-intercept when y = 0) for a positive response in each group to be determined. Analysis revealed at 24 h, the threshold was 0.63 $\mu g \cdot cm^{-2}$ and 1.22 $\mu g \cdot cm^{-2}$ for COL and PLA groups respectively, indicating a dose 1.95 fold greater was required to elicit a positive response in the PLA group. At 48 h, the threshold was 0.51 $\mu g \cdot cm^{-2}$ and 1.06 $\mu g \cdot cm^{-2}$ for COL and PLA groups respectively, indicating a dose 2.09 fold greater was required to elicit a positive response in the PLA group.

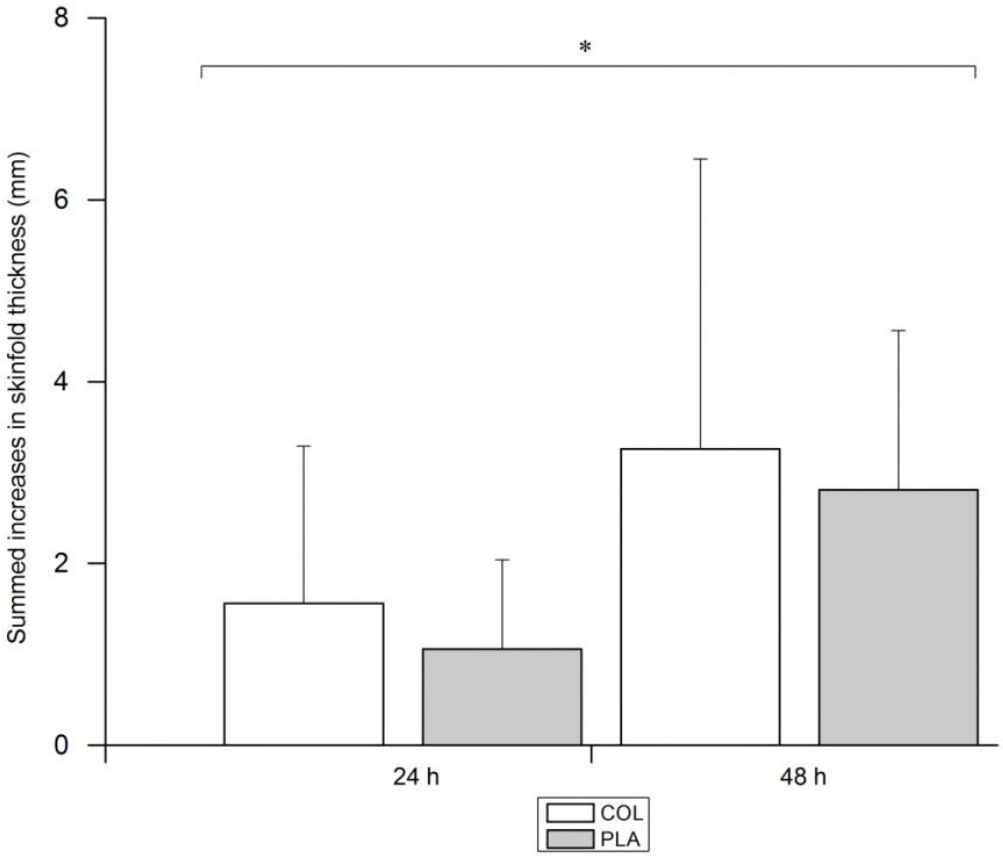

Figure 7.1 Skin responses to DPCP following the completion of exercise prior to induction of contact sensitivity with DPCP in COL and PLA groups. Columns are the means of summed responses to the five doses of DPCP with error bars displaying standard deviation. * Significant main effect of time (increases in summed skinfold thickness from 24 to 48 h) ($p < 0.001$).

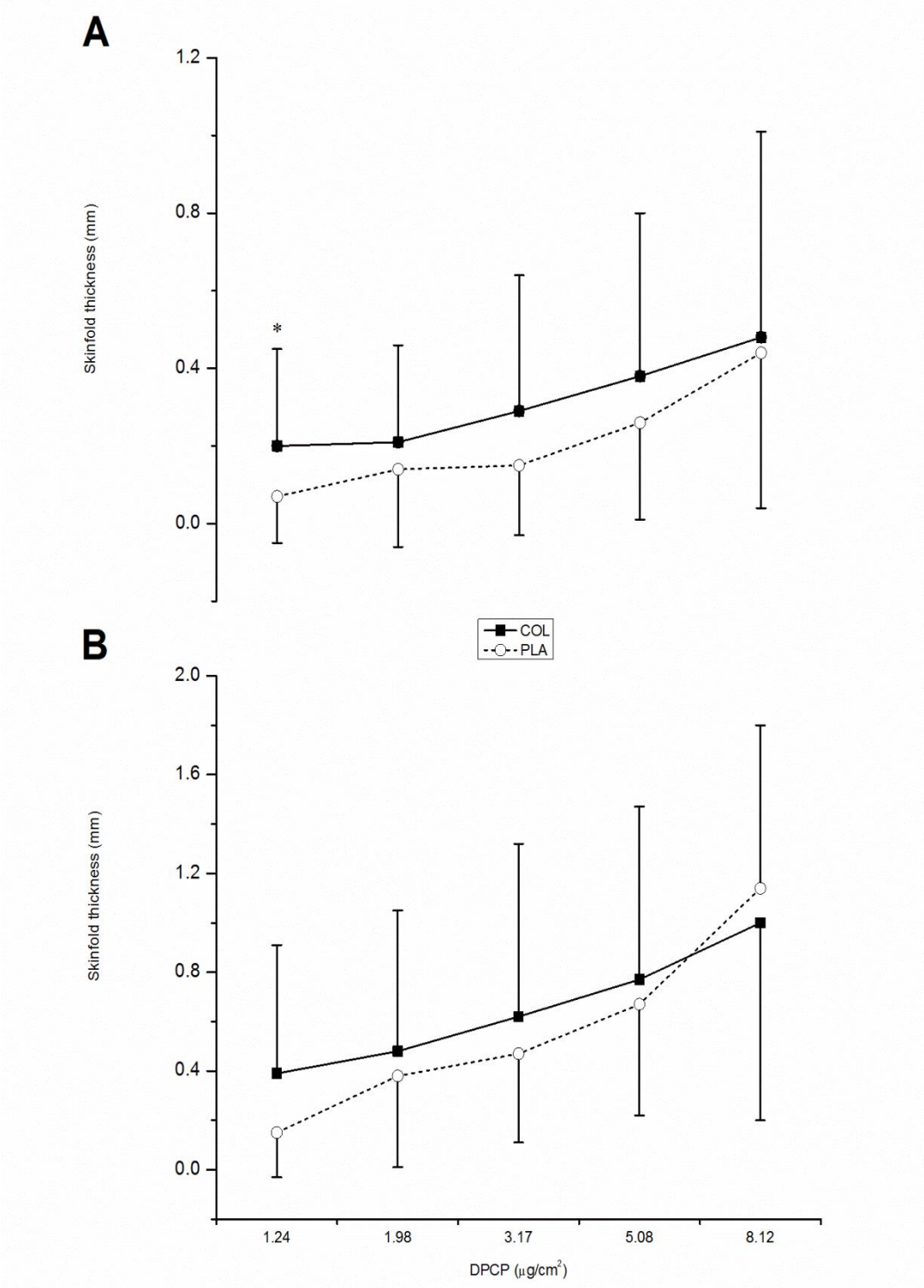

Figure 7.2 Mean skin responses at each of the DPCP doses at 24 h (A) and 48 h (B). Error bars represent standard deviation. *Significant difference between groups at dose ($p < 0.05$).

7.4 Discussion

The aim of this study was to investigate the effects of COL supplementation on the induction of a cell-mediated response to DPCP following prolonged exercise. Previous evidence suggests that completion of prolonged exercise prior to sensitisation to the novel sensitising chemical, DPCP, can impair the induction of antigen-specific memory (Harper-Smith et al., 2011). This provides a robust method to evaluate the effects of purported nutritional countermeasures on exercise-induced immune dysfunction which in this model is assumed to primarily involve T-cell-mediated immunity. In the present study, there was no apparent effects of COL supplementation on the magnitude of cutaneous immune responses (summed skinfold responses) recalled 4 weeks following initial sensitisation to DPCP. This lack of effect in overall reactivity to DPCP (Cooper et al., 1992; Palmer and Friedmann, 2004) was evident at 24 h and 48 h following the recall of antigen-specific memory.

Harper-Smith et al. (2011) also indicated that participation in prolonged exercise prior to primary sensitisation increased the minimum dose (threshold) required to elicit a positive response to DPCP 48 h following the recall challenge. Essentially, the exercise group who suffered the exercise-induced impairment in cell mediated immunity required a DPCP dose of 2.58 fold greater (i.e. lower sensitivity) than the control group to evoke a positive response (i.e. increase in skin oedema). Within the present study, at the same timepoint, a dose of 2.09 greater was required to elicit a positive response in the PLA group compared to COL. Furthermore, these thresholds for positive responses as a dose per unit area (COL: 0.51µg·cm^{-2}; PLA: 1.06µg·cm^{-2}) at 48 h for COL and PLA groups are comparable to the control (resting) and exercise groups of Harper Smith et al. (2011) (control: 0.53 µg·cm^{-2}; exercise:

1.38μg·cm^{-2}). Taken together, this may suggest that COL acts as nutritional countermeasure to prolonged exercise by preventing decrements in the sensitivity of the recall of antigen-specific memory. Such interpretation should be viewed with caution considering that here and within the data of Harper-Smith et al. (2011) the data for minimum thresholds are not exposed to statistical analysis as they must be derived from pooled group data. However, the greater responses to the lowest dose of DPCP at 24 h in the COL group would provide support to a lower threshold with COL supplementation.

There is unequivocal evidence that a threshold effect exists for cutaneous sensitisation (Kimber et al., 1999). It has been substantiated with a number of allergens and contact sensitisers such as nickel and 2-4-dinitrochlorobenzene (Allenby and Basketter, 1993; Friedmann and Moss, 1985). Indeed, with experimental contact dermatitis, there are threshold doses where an allergic state is not clinically elicited despite prior sensitisation (Hostynek and Maibach, 2004). Currently there is no available evidence to suggest that differences in responses to DPCP translate to change in the resistance to other novel antigens (e.g. viruses) in athletes. In other words, clinical relevance of the lower threshold for positive response in COL and greater responses at the lowest DPCP dose (at 24 h) compared to PLA but a lack of effect of COL on the summed oedema responses in the present study is unknown. It is reasonable to suggest that greater immune responses by the host during exposure to a low dose of pathogen would be beneficial for limiting the spread of the infectious agent. Indeed, a major goal of vaccines is to sensitise the host for future exposure to the pathogen (Constant and Bottomly, 1997). It is beyond the scope of the findings of this study to suggest that T-cell mediated responses at lower doses of DPCP are of greater biological

significance than summed responses. Previous studies, however, have demonstrated that COL does reduce the incidence of URI in athletic and immune-deficient populations but generally has no effect on the duration and severity of episodes (Brinkworth and Buckley, 2003; Study 3; Crooks et al., 2010; Patel and Rana, 2006).

It must be acknowledged that the present study design does not allow us to fully establish whether the potential effect of COL on the immune system occurred during the induction and/or the elicitation of cell-mediated immune responses. Within the field of contact sensitisation, the main factor considered to determine the extent of elicitation is the strength of the induction (Hostynek and Maibach, 2004). In addition, the stronger the degree of induction, the lower the dose that sensitised individuals will react to upon any recall challenge (Friedmann, 2007). Given the extent of depression in the induction phase of T cell memory following prolonged exercise (Harper-Smith et al., 2011), it is reasonable to suggest that this is the period that would have benefited most from a nutritional countermeasure such as COL. Evidence to date (Davison and Diment, 2010; Study 1, 3) suggests that the effects of COL may be more apparent in the early recovery period following prolonged exercise. The immunoprotective effects of COL within a model of exercise-induced immune dysfunction thus far in humans have only been demonstrated within measures of neutrophil function. Biswas et al. (2007) did, however, demonstrate that COL can differentially affect *in vitro* stimulation of human PBMC by enhancing IFNγ production during weak antigenic stimulation but inhibiting IFNγ response during strong antigen stimulation. Whether such findings provide and explanation to the findings of our study remains speculative at present.

Although T cell infiltration plays a central role in the orchestration of the DTH response, this is initiated by the non-specific ‘sensitivity’ of other local (dendritic, Langherhans) cells that respond to perturbations induced by the antigen (Friedmann et al., 1993). In addition to the induction phase, recent evidence suggests that the recruitment of antigen-primed CD8+T cells into the elicitation site may also depend on the infiltration and chemoattractants released by inflammatory cells (e.g. neutrophils) (Engeman et al., 2004; Kish et al., 2012). The lack of measurement of circulating cytokines within this study limits any discussion on the effects of COL on signals that may have triggered the migration and maturation of these innate cells. Such findings may have reflected possible tissue priming effects that resulted in enhanced immunosurveillance and explain the greater responses at lower concentrations of DPCP in the COL group. Future studies should explore any effects of COL supplementation on immune parameters prior to induction and elicitation separately in order to determine potential mechanisms. It also would be prudent for investigators to measure oedema prior to the 24 h timepoint if an intervention is proposed to improve the sensitivity of responses.

The mechanisms underpinning the effects of prolonged exercise on responses to DPCP are still unclear. The present study observed no effects of COL on the magnitude of skin oedema following recall of antigen memory. Harper-Smith et al. (2011) suggested that reductions in both induction and elicitation of such responses may be due to stimulation of the sympathetic nervous system and HPA axis (Dhabar, 2002; Dhabar and McEwen, 1999). The increase in total and differential leukocytes within the present study is in line with such prolonged exertion where changes are well recognised to be due to increases in circulating concentrations of catecholamines and cortisol (Anane et al., 2009; Atanackoviv et al., 2006; McCarthy

and Dale, 1988; Nieman, 2001). The similar leukocyte trafficking between groups suggests that they were exposed to a matched physiological stressor and provides further evidence that COL does not mediate immune responses to exercise via such mechanisms. Given that carbohydrate supplementation during prolonged exercise has been shown to attenuate perturbations in stress hormones and/or *in vitro* measures of immune function (Davison and Gleeson, 2005; Lancaster et al., 2005a; Scharhag et al., 2002), use within the current experimental model warrants investigation.

In summary, COL may affect the sensitivity of the *in vivo* response to a novel antigen but it has no effect on the overall magnitude of such responses. Further research is required to determine the clinical implications of such effects and whether they may provide further mechanistic explanations of the decrease in the incidence of URI during COL supplementation within athletic and immune deficient populations.

Chapter 8. General Discussion

The series of studies presented in thesis (Study 1-5) were aimed to determine the effects of COL supplementation on the human immune system and risk of URI. The primary focus of the initial studies (Study 1 and 2) were to examine the influence of COL on neutrophil responses to a physical stress model (2.5 h of prolonged cycling) expected to result in exercise-induced immune dysfunction. These involved either consumption of COL on the day of exercise (1 h and immediately pre-exercise and mid-way through exercise) or supplementation for 28 days prior to the exercise trials. The secondary outcomes of these studies were also to investigate the effects of COL on mucosal immune responses to the prolonged cycling. This allowed for the independent investigation of acute and longer term supplementation of COL.

As it was anticipated that any effects of COL were likely to be related to the absorption of bioactive components/derivatives which become bioavailable following digestion, a further study was designed with a chronic supplementation period (12 weeks) to identify whether immune-enhancing properties would become apparent at rest. Given the opportunity of a sufficient window to identify differences in incidence of illness, the primary outcome in this study was incidence of URI. Due to the nature of the previous studies, a group of participants who performed regular endurance training were chosen as it was considered that they would expose themselves, regularly, to similar stressors imposed in our physical stress models and hence provide greater scope for an intervention with a nutritional countermeasure.

The aetiology of URI in athletes is an on-going investigation with infectious and non-infectious causes being explored. One such risk factor is latent herpes viruses. Therefore, in the same cohort as study 3, study 4 investigated whether susceptibility

to URI in seropositive or seronegative to EBV was affected by COL supplementation. To reflect a ‘normal situation’, minimal efforts were taken to modify the diet of participants. The common approach across all of these studies was to request for participants to arrive fasted to the laboratory on the day of all visits which involved measurement of immune parameters. Due to the crossover design of study 1, this study also involved a replication of diets in the day prior to trials.

In the concluding experimental chapter (Study 5), additional standardisation of dietary intake was used. This involved prescribed food and drink intake in the 24 h prior to and on the day (breakfast and post-exercise snack) of the main exercise trial. Essentially, this was not an addition to provide any greater dietary standardisation than previous thesis studies *per se* but it was to follow the methodology of a previous study by Harper-Smith et al. (2011). This latter study demonstrated a depression in the induction of an *in vivo* immune response to a novel antigen (DPCP) in a group who performed prolonged exercise compared to seated rest (control) prior to sensitisation. Study 5 was designed to investigate the effects of supplementing the dietary intake of Harper-Smith et al. (2011) with COL or PLA for 28 days prior to and following the prolonged exercise. Any differences between groups would be suggested to be due to differences in the induction of immune responses (DTH) to an antigen following exercise or changes at rest (elicitation) following COL supplementation. With the expectation that the primary factor on the responses would be the sensitisation period (immediately following the exercise), matching the protocol to that of Harper-Smith et al. (2011) would provide an indicator of how groups compared to the control group of the latter study (i.e. extent of immunodepression). A summary of the effects of COL on all immunological

outcomes within this thesis is provided in Table 8.1 with further discussion on the specific measures in the remaining sections of this chapter.

Table 8.1 Effects of COL supplementation on measured immune parameters within the thesis.

	Supplementation		
	Acute (pre- and during exercise)	4 weeks (pre-exercise)	12 weeks (rest)
Bacterial-stimulated elastase release	+	=	NA
Neutrophil oxidative burst	+	+	=
Salivary bacterial load	NA	NA	+
Salivary SIgA	=	=	=
sLac	=	=	=
sLys	+	=	=
T-cell-mediated contact hypersensitivity	NA	+	NA

+ Benefit in COL compared to PLA (trial or trial × interaction: $p < 0.05$); = No difference between COL and PLA ($p > 0.05$): NA Parameter not analysed in study.

8.1 Bovine colostrum and exercise-induced leukocytosis

It is well established that leukocytosis occurs during and immediately post-exercise with the observed changes being dependent on the exercise intensity and duration (Gleeson, 2007). In support of previous evidence (Davison et al., 2012; Henson et al., 2008; Robson et al., 1999b), the prolonged bouts of exercise (2-2.5 h) within this thesis induced significant increases in total and differential leukocytes. Such absolute changes in leukocyte trafficking and other indicators (e.g. increases in neutrophil:lymphocyte) demonstrate the extent of the physiological stress placed on the immune system (Nieman, 1998). The magnitude of these changes could be considered lower than previous studies which most likely reflect differences in training status of participants and/or intensity (cycling) and/or duration (running) of exercise (Davison and Gleeson, 2005, 2006; Davison et al., 2007, 2012; Henson et al., 2008; Laing et al., 2005; Nieman et al., 1998).

Nevertheless, the observed leukocytosis is likely to be due to a combination of the activation of the sympathetic nervous system and HPA axis as well as the increased cardiac output and subsequent shear stress (increased blood flow) within blood vessels during and following prolonged exercise (Anane et al., 2009; Atanackoviv et al., 2006; Nieman, 2001). As shown previously (Carol et al., 2011; Davison and Diment, 2010), there was a lack of effect of COL on exercise-induced leukocytosis. Given the well accepted role of increases in catecholamines and cortisol in leukocyte trafficking, the findings of the present thesis may provide further evidence that any effects of COL on immune function do not appear to be related to an attenuation of such physiological changes during exercise.

There was also no evidence of significant changes in leukocyte counts (at rest rather than exercise) following 12 weeks of COL supplementation. Although attenuation of decreases in cytotoxic/suppressor T cells have been observed previously (Shing et al., 2007), this was during and following an intensified training period. Within the present thesis, the measurements were taken in a cross-sectional manner where participants had avoided exercise for at least 24 h. Generally, evidence suggests that leukocyte counts measured in a resting state, as here, are similar between athletes and non-exercising controls (Walsh et al., 2011b), thus it is not surprising to observe no differences between COL and PLA groups who were matched for training volume (duration and intensity).

8.2 Bovine colostrum and neutrophil responses

Despite regular exercise training having minimal effects on neutrophil counts, the leukocytosis that occurs following prolonged exercise is primarily mediated by a rise in circulating neutrophils (Walsh et al., 2011b). This neutrophilia is believed to be partly due to an immediate and delayed release of neutrophils from marginated pools within the body and bone marrow respectively (Allsop et al., 1992; McCarthy and Dale, 1988; Peake, 2002). The circulating post-exercise neutrophil population are suggested to include a greater proportion of immature cells (e.g. band cells) compared to pre-exercise which has been implicated to play a role in the decrease in neutrophil function (on a per cell basis) following prolonged exercise (Berkow and Dodson, 1986: Hetherington and Quie, 1985; McCarthy et al., 1991; Yamada et al., 2002). For this reason, the most effective nutritional countermeasures to exercise-induced neutrophil dysfunction are those which can attenuate leukocytosis and neutrophilia (i.e. carbohydrate) (Nieman, 2008). However, based on previous *in vitro*

co-culture studies (Sugisawa et al., 2001, 2002, 2003), the working hypothesis within this thesis was that COL supplementation would have direct effects on neutrophil function rather than any effect on their trafficking in response to exercise.

In line with other leukocyte subsets, there was no effect of COL on neutrophil counts at rest or following exercise. However, in support of a previous study (Davison and Diment, 2010), study 1 suggested that COL supplementation improves the recovery of neutrophil degranulation following prolonged exercise. In contrast to Davison and Diment (2010) the present thesis also explored the effects of COL supplementation on stimulated neutrophil oxidative burst. Study 1 is the first study to show that acute supplementation of COL can result in greater fMLP-stimulated oxidative burst compared to PLA and enhance the recovery of neutrophil degranulation following prolonged exercise. This supports studies of short term *in vitro* culture which demonstrated that COL possesses the ability to prime neutrophil function upon subsequent activation (Benson et al., 2012; Sugisawa et al., 2001, 2002, 2003). Sugisawa et al. (2003) proposed that 'priming' induced by COL is due to low molecular weight proteins (e.g. proteose peptones). The enhancement of fMLP-stimulated oxidative burst of neutrophils measured at rest (i.e. BAS) and post-exercise by *in vitro* pre-incubation with plasma obtained following (acute) COL consumption does provide preliminary evidence to suggest that priming or immune enhancing components become bioavailable *in vivo.*

Study 1 was directed by a pilot study where PMA-stimulated oxidative burst was enhanced 1 h after COL consumption in resting healthy participants. There was, however, a lack of effect of COL on PMA-stimulated oxidative burst at post-exercise timepoints in study 1 and even pre-exercise (1 h post consumption), unlike what was

seen in the pilot work of that study. This may highlight a multi-faceted mechanism on neutrophil oxidative burst which is affected by the perturbations during and following prolonged exercise. For example, the priming of neutrophils is not an irreversible process (Kitchen et al., 1996; Summers et al., 2010), thus an effect of 'depriming' on responses to agonists such as PMA following exercise should be determined. Changes in neutrophil effector functions with prolonged exercise have been suggested to depend on the balance of immunosuppressive (e.g. 'refractory period') and immunostimulating factors (e.g. priming agents) (Peake, 2002)

The role of low molecular weight peptides remains preliminary at present but it must be recognised that as cytokines (GM-CSF, G-CSF, IL-8, TNFα) which are known to regulate neutrophil functions have not been measured (Elbim et al., 1994; Khwaja et al., 1992; You et al., 1991), the role of mediators other than the proposed components (i.e. proteose peptones) cannot yet to be dismissed in human studies. Pancreatic enzymes produce numerous potential biologically active peptides from the digestion of COL (Jorgensen et al., 2010). For example, although IgG (a major indicator of COL supplement quality, Gulliksen et al., 2008) within non-digested COL is unlikely to pass into circulation it may act as precursor to other immune modulatory components (metabolites). Tuftsin, a small (~500 Da) tetrapeptide produced from the digestion of the Fc heavy chain of IgG has been shown to enhance phagocyte (i.e. neutrophil) chemotaxis and oxidative burst (Najjar and Nishioka, 1970; Siemion and Kluczyk, 1999; Werner et al., 1986; Wu et al., 2012). Additionally, evidence that components of bovine whey protein extracts (β-lactoglobulin, α-lactalbumin) can survive gastric digestion and prime neutrophil responses to fMLP (Rusu et al., 2009) demonstrates the range of potential

immunomodulatory components within COL and related preparations (Severin and Wenshui, 2005).

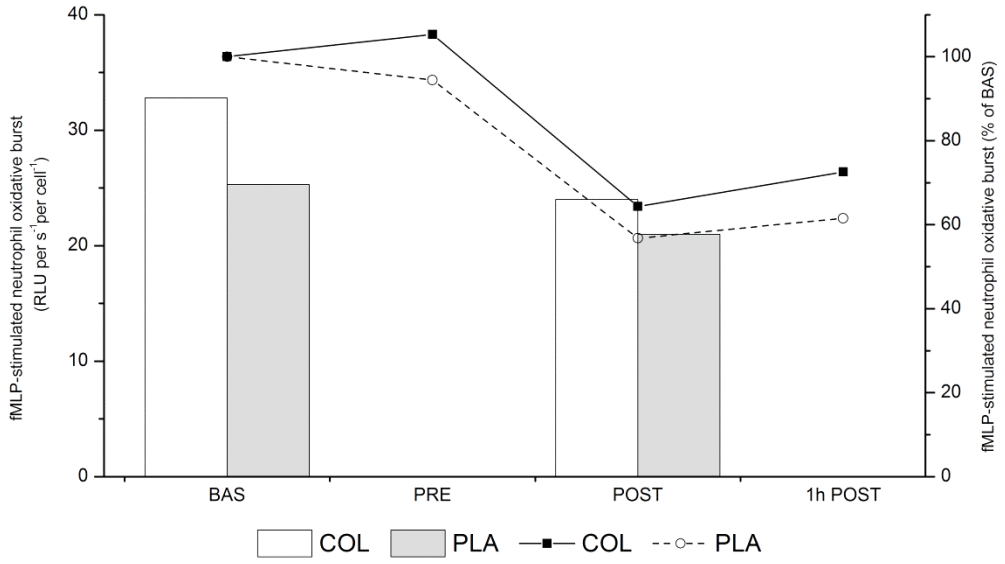

Figure 8.1. Schematic representation of the effects of acute COL supplementation. Incubation of plasma obtained following 1 h of COL or PLA consumption with pre- or post-exercise blood (shown as bars) and the responses of the same subset of participants to the experimental trials (acute COL supplementation) (shown as line and symbol) of study 1.

Nevertheless, as longer term supplementation (4 weeks, Study 2) had greater effects on fMLP oxidative burst than acute supplementation (Study 1) it may suggest that a long term exposure to priming agents via full absorption and/or accumulation of COL leads to the most effective use of the nutritional intervention. However, it must be acknowledged that there remains to be a lack of acute COL supplementation studies to conclude a greater effect with chronic supplementation. It is reasonable to suggest that acute supplementation would require a higher dosage of COL, future studies should expand on the design of the pilot study in this thesis to determine the optimal timing and dosage of acute and/or chronic supplementation of COL prior to exercise. Such studies are likely to identify the kinetics at which the priming components and/or other metabolites involved in enhancement of neutrophil function appear and

leave the circulation following COL supplementation. The evidence gathered in this thesis does at least suggest that the effects of a large acute dose or longer term daily supplementation of COL on neutrophil responses are not related to an up-regulation of antioxidant defences as found previously with other supplements with antioxidant components (Davison and Gleeson, 2007).

Furthermore, it seems that the effects of COL on neutrophil function are most apparent under circumstances of greatest immunodepression. This may be supported by the lack of decrease in neutrophil degranulation post-exercise (no subsequent effect of COL) in study 2, whereas in Davison and Diment (2010), COL enhanced the recovery of this parameter 1 h following exercise compared to a sustained decrease in the PLA group. The lack of effect of 12 weeks of COL supplementation on fMLP-stimulated oxidative burst in active males at rest may also support a hypothesis that the effects of COL are more apparent during exercise-induced immune dysfunction. It is worthy to note that a paired t-test analysis of the COL group in study 3 did demonstrate an increase in fMLP oxidative burst ($p = 0.008$) across the 12 weeks. The lack of significant difference between the groups may have resulted from a lack of statistical power.

In general, the effects of COL on neutrophil responses, in particular with exercise-induced immune dysfunction, appear to be receptor dependent (fMLP oxidative burst, bacterial-stimulated degranulation). The physiological relevance of these responses is that the investigated agonists (fMLP, LPS) are known to be released at sites of inflammation (as a result of infection or tissue damage) (Carp, 1982; Mantovani et al., 2011; Marasco et al., 1984; Tennenberg and Solomkin, 1990). The importance of neutrophil function is also exemplified within individuals who are prone

to recurrent bacterial and fungal infections as a result of inherited defects in such pathways (Summers et al., 2010).

Shing et al. (2007) observed a minor enhancement of cell surface receptor (CD89) expression on neutrophils at rest during 5 consecutive days of high-intensity training but not following exercise in the COL-supplemented group. Despite impaired receptor expression resulting in decreased oxidative burst activity in clinical settings (e.g. Lin et al., 1994; Rosenbloom et al., 1995), investigators have failed to find any consistent association following exercise (Peake et al., 2004). Neutrophil priming has been shown to increase the surface expression of the receptor for fMLP but there has been conflicting evidence to whether this results in increased oxidative burst responses (Condliffe et al., 1996; Elbim et al., 1994; O'Flaherty et al., 1991). Furthermore, cell surface receptor expression is considered to have limited value as a marker of human immune modulation within intervention studies (Albers et al., 2005). Although this may be relevant for receptor expression, little is known about receptor function and coupling to intracellular signals (Mooren et al., 2001). Therefore, the inclusion of measures of cell surface receptors within future studies should provide a more detailed investigation of the mechanisms underlying the effects of COL on neutrophils.

8.3 Bovine colostrum and mucosal immunity

Given the potential for COL to promote the integrity of the human epithelium (He et al., 2001), stimulate GALT and act as a source of growth factors (TGFβ) considered to play a role in mucosal biosynthesis and secretion of Ig (Chen et al., 1990), it may be expected to see benefits on mucosal immunity. The secondary outcomes of study 1, 2 and 3 were to determine any effect of COL on mucosal parameters. As IgA is

the most predominant mucosal Ig, the majority of previous investigations had focused on this parameter (Bishop and Gleeson, 2009; Walsh et al., 2011b). Although there was a decrease in saliva SIgA:osmolality, the concentration and secretion rate of salivary SIgA remained unchanged following prolonged cycling in this thesis. The lack of change following exercise agrees with some (MacKinnon and Hooper, 1994; Nieman et al., 2003; Sari-Sarraf et al., 2006) but not others (Nieman et al., 2002a; Palmer et al., 2003; Tomasi, et al., 1982). It seems that the combination of high-intensity and long duration exercise that has the most significant impact (i.e. depressive) on salivary SIgA concentration (Mackinnon, 1996; Nieman et al., 2002a). Therefore, it would be reasonable to suggest that the cycling protocol used in the exercise studies in this thesis was not of sufficient intensity and/or duration to induce dysfunction of all mucosal parameters and therefore identify an effect of COL on salivary SIgA.

In contrast to directly preventing a decrease post-exercise, an alternative way in which an intervention could enhance salivary SIgA would be to elevate concentrations at rest (i.e. pre-exercise). Some investigators had found increases in resting saliva IgA (Appukutty et al., 2010; Crooks et al., 2006; Mero et al., 2002) concentrations following COL supplementation but others have failed to support such findings (Crooks et al., 2010; Davison and Diment, 2010; Patiroglu and Kondolot, 2013; Shing et al., 2007, 2013). The variability in study design makes interpretation of the effects of COL difficult, highlighting similar issues with investigations of other supplements in the area of exercise immunology (Moreira et al., 2007). Mero et al. (2002) found a 33% increase in resting salivary IgA concentration, following 2 weeks of COL supplementation where dosage was split across the day (4 × 5 g). Such changes in Mero et al. (2002) may be due to the multiple dosages having a

cumulative stimulatory effect on IgA synthesis and/or secretion. The lack of macronutrient and caloric matched PLA in Mero et al. (2002) does confound the findings. The findings of no change in salivary SIgA with acute consumption of COL prior and during the exercise (Study 1), would not support any direct effect of COL on production or release. Given that the half-life of IgA is ~ 3-6 days (Miletic et al., 1996), it may be suggested that longer term supplementation may be required to have a significant impact on the SIgA pool. However, a similar total daily COL dosage as Mero et al (2002), split twice a day, but taken for a longer period (4 weeks) had no effect on resting salivary SIgA concentration in study 2 or Davison and Diment (2010).

Crooks et al. (2006) found a 79% increase in resting median IgA concentration in saliva but this effect was only significant following 12 weeks of supplementation and not at 4 or 8 weeks. The authors of this study did, however, report a large variability in IgA concentrations across participants. Study 3 observed no significant effect of 12 weeks of COL on resting salivary SIgA expressed as total concentration, secretion rate or relative to osmolality. When comparing the baseline to the 12 week timepoint only, there were minimal changes in IgA concentration or IgA:osmolality in the COL group. There was, however, a 23% increase in salivary SIgA secretion rate compared to a decrease in secretion rate in PLA (13%). There was also a 17% and 14% decrease in PLA concentration and IgA:osmolality respectively at 12 weeks. The clinical relevance of such changes are unclear as they are somewhat lower than the 40% decrease in salivary SIgA that was associated with a 50% risk of contracting URI in a group of yachtsmen (Neville et al., 2006). Nevertheless, this would suggest a stabilising effect of COL rather than an enhancement of salivary SIgA per se. As large variability in salivary SIgA may again be a confounding factor here, further

longitudinal investigations involving athletes who partake in greater weekly training loads (e.g. Gleeson et al., 2012b) and the same type of sport is required (as in Crooks et al., 2006) to clarify the effects of COL.

In addition to SIgA, saliva contains an abundance of AMPs which have received little attention to date in relation to response to prolonged exercise (Walsh et al., 2011b). In a study involving a prolonged exercise bout (2 h cycling at ~64% $\dot{V}O_2$ max), Davison and Diment (2010) found that 4 weeks of COL supplementation (20 g·day^{-1}) reduced the exercise-induced decrements in sLys concentration and secretion rate (immediately post-exercise). A similar pattern was found with acute consumption of COL in study 1. The effects occurred despite the lack of significant exercise-induced decrease in sLys that was observed in Davison and Diment (2010). Such findings may provide further evidence to the aforementioned proposals regarding COL and priming of the effector responses of neutrophils. Studies (Davison and Diment, 2010; Study 1) which have found benefits on sLys thus far have also seen enhancements in neutrophil degranulation responses. The lack of influence of COL on sLys and neutrophil degranulation in study 2 may provide indirect evidence to support this hypothesis. As neutrophils are considered an important source of sLys, further research is required to determine whether changes in some salivary AMPs with COL and/or exercise are due to differences in neutrophil activation in the circulation and oral cavity.

In contrast to study 1, no other studies in this thesis reported any beneficial effects of COL on sLys or sLac. There are a limited number of investigations that have assessed the change of AMPs during regular training (He et al., 2013a; West et al., 2006). sLys or sLac remained unchanged or increased over the 12 week period in

study 3. The changes in the sLac at rest are consistent with exercise-induced changes in study 3 and other investigations of sLac and other AMPs with similar sources (e.g. LL37) (Davison et al., 2009; Gullim et al., 2013; Usui et al., 2011). Compared to salivary SIgA, the functional significance of changes in sLac is unclear at present but it may reflect an inflammatory response to exercise-induced damage to mucosal surfaces (Davison et al., 2009; Usui et al., 2011). It must be recognised that the studies within this thesis have only investigated a narrow range of an available wide range of AMPs within the oral cavity (West et al., 2006). Despite the well accepted role of AMPs in oral health and infection (Dale et al. 2006; Putsep et al., 2002; Tanida et al., 2003; Tao et al., 2005), there remains few investigations of their contribution towards protection against URI. Furthermore, the existence of synergism between these peptides (De Smet and Contreras, 2005), highlights the need for an integrated, holistic immune marker which may account for changes in any of the AMPs.

The oral cavity is colonised by a diverse range of commensal microbes (Bosch et al., 2013; Watson et al., 2006). As the surfaces of the oral cavity are bathed with saliva, the oral fluid is considered to provide a 'fingerprint' of the vast range of the resident microorganisms (Boutaga et al., 2007; Dewhirst et al., 2010; Fabian et al., 2008; Li et al., 2005). The commensal bacteria are tightly regulated by local immune factors including but not limited to the synergic arsenal of AMPs (Boman, 1995). The importance of such defences has been demonstrated within *in vitro* models whereby reduced expression of AMPs leads to changes in bacterial colonisation (Bals, 1999; Liu and Modlin, 2008). Furthermore, perturbations in the balance of the microbiota on the mucosal surface can lead to overgrowth and further amplification of microbes

as seen in conditions of dysregulated innate immunity (e.g. aging, obesity) (Amar and Leeman, 2013; Krone et al., 2013).

Study 3 is the first study on salivary microbiome in participants involved in regular training, showing increased bacterial load during winter months which was limited with COL supplementation. It is proposed that over the course of the 12 weeks the COL group suffered fewer incidences of transient immune perturbations, and/or smaller or shorter disturbance (in response to training and/or seasonal variation), which may have limited conditions for changes in the salivary microbiome to occur. The lack of direct antimicrobial effect of COL within study 3 may provide additional support that these effects are related to the immune-modulatory properties of COL. As the oral cavity provides the main route of transmission for pathogen entry, saliva bacterial load may provide to be a sensitive *in vivo* marker of immunity and susceptibility to URI.

8.4 Bovine colostrum, delayed-type hypersensitivity and upper respiratory illness

It is apparent that the magnitude of change in immunity immediately following each bout of strenuous exercise may have more clinical significance than training-induced alterations in resting immunity (Abbasi et al., 2013; Nieman et al., 1994; Pedersen and Bruunsgaard, 1995). The importance of *in vivo* measures of immunity to determine the clinical relevance of an intervention have been recognised (Albers et al., 2005). Contact sensitisation can be used as an experimental model to determine the effects of interventions on the induction and elicitation phases of *in vivo* T-cell-mediated immune response (Albers et al., 2005; Friedmann, 2007; Palmer and Friedmann, 2004). Harper-Smith et al. (2011) demonstrated that participation in a

single bout of prolonged moderate intensity exercise compared to rest significantly reduced both the induction as well as the elicitation of the cell-mediated immune response to a novel antigen, DPCP.

Study 5 showed a lack of effect of COL supplementation compared to PLA on the magnitude of the immune response (measured by oedema) to DPCP where induction of T-cell-mediated immunity was preceded by prolonged exercise (as in Harper-Smith et al., 2011). However, there was evidence of an increase in the sensitivity of the response at 24 h and 48 h following the recall of immune memory to DPCP whereby a lower dose was required to elicit a positive response in the COL group. Such findings may support a similar priming effect on the host that has been proposed for other immune measures (e.g. neutrophil oxidative burst) but the mechanisms of how COL may be having an effect on the sensitivity of the responses are unclear at present. Nevertheless, it is reasonable to suggest that if COL does modulate human immunity in such a way that it responds to lower dose of a novel antigen (e.g. viral antigen) it would be beneficial to the host. This is particularly relevant for the recovery window (e.g. 24 h) following prolonged exercise where athletes may be exposed to pathogens on a regular basis during training and competition schedules.

Further research is required to determine the use of this DPCP experimental model as an indicator of susceptibility to URI. Furthermore, as most of the immunoprotection with COL in an exercise setting thus far has been limited to innate/mucosal parameters, this experimental model (purely a T-cell-mediated response) may not be at the most relevant site to indicate the extent of effectiveness of a COL intervention. An increasing number of animal inoculation studies are

showing that COL can increase the amelioration of bacterial (e.g. *Streptococcus*) and viral (e.g. Influenza A) infection due to up-regulation of local immune defence (e.g. mucosal tissue within the respiratory tract) (Benson et al., 2012; Uchida et al., 2012; Xu et al., 2013). Furthermore, the findings from human studies demonstrating an increased vaccination response and/or subsequent lower number of episodes of illness with COL supplementation may suggest the inclusion of *in vivo* makers of humoral immunity are warranted in future investigations (Cesarone et al., 2007; He et al., 2001).

In support of previous evidence (Brinkworth and Buckley, 2003; Crooks et al., 2010), COL supplementation reduced the susceptibility to URI (Study 3). In contrast to previous studies, study 3 indicated that the beneficial effect of COL may become apparent with shorter supplementation (< 4 weeks). Although findings from some immune parameters (e.g. fMLP oxidative burst) suggest that longer term supplementation (e.g. 4 weeks) of COL has greater effects, it seems that there may also be clinical relevance to shorter term supplementation. It was, however, concluded in this study that the benefits of COL are most apparent during periods of greatest prevalence of URI. This may have relevance to previous discussions within this thesis. It has been suggested that COL may reduce the duration and/or severity of the 'open window' following acute, prolonged/strenuous exercise. During a longitudinal study (e.g. Study 3), such effects may reduce the overall risk of URI for each individual in the COL group.

As shown by others, there was no effect of COL on the duration and/or severity of reported URI episodes (Brinkworth and Buckley, 2003; Crooks et al., 2006, 2010). Patiroglu and Kondolot (2013) did report a reduction in the severity of URI with COL

supplementation but this did involve a cohort of children who were clinically immune (IgA) deficient at rest. Further evidence is required to determine whether COL would affect duration and/or severity of URI in athletes who are exposed to a greater load of training stress (i.e. than participants used in study 3), hence are at risk for greater immune-dysfunction. At present, however, the lack of pathogen identification within human studies of COL and risk of URI cannot dismiss other underlying mechanisms related to 'non-infectious' stimuli. Animal studies report that bovine milk products (e.g. whey protein extract) via TGFβ activity can increase the population of T^{reg} cells resulting in reduced $T^{h}2$ related cytokine production and airway inflammation/hyperresponsiveness (Chen et al., 2013). It would be prudent for future investigations to identify the effects of COL on the typical polarisation of T^{h} cells toward the $T^{h}2$ phenotype with chronic exercise training and hence the potential pathways of non-infectious, inflammatory causes of URI (Bermon, 2007, Lakier-Smith, 2003; Steensberg et al., 2001)

Study 4 did report a fewer number of URI days in participants in a COL group who were EBV^{+} compared to EBV^{-} counterparts. This data should be interpreted with caution as the outcome does depend on the duration of each URI. Such influence could be heightened considerably in a small group of participants and/or low number of illnesses. Nevertheless, this provides a prudent area of research given that latent herpesviruses may also influence the susceptibility to URI (He et al., 2013b). This potential cumulative effect of COL and EBV status on URI duration reported in study 4 alongside the findings of He et al. (2013b) does contradict earlier hypotheses which suggested that prior infection with herpesviruses may have a detrimental effect on health of the athlete. There was no apparent enhancement in immune parameters taken at rest in study 4 to suggest a cumulative effect but the findings

are limited with an absence of such investigation during URI episodes. Despite a potential role of prior EBV infection with this measure, it is worthy to note that the effects of COL on incidence of URI compared to PLA was evident whether participants were seronegative or seropositive or if both groups together were considered.

8.5 Summary and conclusions

The general findings in this thesis provide convincing evidence that 1 h to 4 weeks of COL supplementation can have beneficial effects on human immunity (e.g. neutrophil oxidative burst) during exposure to an acute physical stressor (prolonged exercise) and lower the incidence of URI during regular endurance training. As it may be expected, such effects become more evident when there is greater immunodepression or prevalence of illness. Study 1 and study 2 of this thesis have substantiated previous findings from our laboratory (Davison and Diment, 2010) that COL can augment parameters of innate immunity. These studies, in combination, have alluded to the possible mechanisms underlying the effects of COL on human immune function. It is suggested that the mechanisms, in part, are due to small bioactive components that survive digestion and/or are produced upon digestion of COL. Study 1 suggests that these effects are apparent 1 h after COL consumption but a longer period of supplementation (4 weeks, study 2) leads to greater effects, possibly due to greater absorption and/or accumulation of these components and hence greater effects as a nutritional countermeasure. Taken together, the findings of these studies suggest that COL primes the effector functions of neutrophils for subsequent activation by bacterial stimulation.

During winter months (Study 3), an increase in salivary bacterial load in a PLA group (likely triggered by seasonal effects and/or repeated/frequent 'open window' of exercise-induced immune dysfunction during regular training) but this increase was blunted by COL supplementation. Although there was a lack of effect of COL on isolated immune measures at rest (fMLP oxidative burst, salivary SIgA, sLac, sLys), it is proposed that salivary bacterial load represents a relevant integrated, holistic marker of *in vivo* (innate) immunity in the oral cavity which may be a further mechanism underlying immune-modulation and risk of URI with COL supplementation. Although COL supplementation did reduce the incidence of URI in a cohort of active males (Study 3), there was a lack of effect on duration and severity of episodes. Investigation of other potential risk factors (EBV serostatus) did not influence susceptibility to incidence of URI (Study 4). However, comparison of EBV^+ and EBV^- males did indicate that prior infection with herpesvirus (EBV^+) and COL supplementation may together reduce the duration of URI episodes.

In a model of contact hypersensitivity, COL did not influence the magnitude of T-cell-mediated responses to a novel DPCP where induction of immune memory took place following an exercise bout shown previously to trigger a ~ 50% fall in responses (Harper-Smith et al., 2011). There was, however, evidence that COL may lower the threshold for an immune response to a previously encountered antigen. Such effects may be related to the aforementioned mechanisms and/or increase in the 'sensitivity' of other inflammatory cells which respond in the immediate period following challenge by a novel antigen.

8.6 Practical implications

The series of studies in this thesis aimed to investigate the use of a nutritional supplement which was proposed to act as countermeasure to prolonged exercise-induced immunodepression and reduce the incidence of URI during regular exercise training. Based on the findings the following practical implications have been developed.

1. Consumption of COL in the morning (1 h) prior (35 g) to and 5 g during a prolonged (> 2.5 h) bout of cycling can have beneficial effects on immune function. This is likely to be most effective during an intensified period of training where there are cumulative bouts of strenuous exercise.
2. Longer periods (e.g. 4 weeks) of COL supplementation (20 g per day) have greater effects on immune function in those participating in prolonged cycling. Due to larger effects than acute supplementation, this practice may be suitable for such exertions that are completed in a low carbohydrate (i.e. fasted state).
3. Daily COL supplementation (20 g per day) reduces the incidence of upper respiratory illness. Such immunoprotection is likely within 4 weeks and will continue to be of further benefit, particularly in the periods of greatest prevalence of URI (i.e. winter).

8.7 Future directions

1. Following the findings of study 1, further research is required to define the optimal timing and/or dosage of COL to have the greatest effect on exercise-induced modulation of neutrophil function. Furthermore, it remains unclear whether the supplementation protocol of study 1 could be combined with that of study 2 to have additive effects on neutrophil responses. In relation to the practical

implications discussed above, it would be advantageous to identify how long the immune enhancing effects of COL last for, following consumption.

2. The pilot study prior to study 1 suggested that acute COL consumption enhances neutrophil responses to stimulation by PMA. Within an exercise stress model, the effects of COL were most apparent in receptor-dependent responses of neutrophils (i.e. not PMA). Further research is required to determine the effects of acute consumption of COL at rest and following exercise in order to identify whether mechanisms are independent of activation of PKC and limited to signal transduction distal to cell surface receptors. Investigations of any changes in receptor expression on the surface of the neutrophils are likely to clarify possible mechanisms of COL supplementation.
3. The findings of study 1 suggest that the effects of COL are at least partly due to the bioactive peptides (or other substances) that become bioavailable upon consumption of COL. Further research should attempt to determine such components through a combination of metabolomic and proteomic approaches. Based on the available evidence, future studies should perform analyses of plasma/serum for molecules within the range 1.4-10 kDa. Upon identification, such analyses may allow for the amplification or separation of the components from COL to identify whether immune enhancing effects (e.g. priming of neutrophil oxidative burst) of COL that occur *in vitro* also exist *in vivo* and not initiated by multiple mechanisms.
4. The lack of effect of COL on salivary SIgA at rest or in response to exercise in this thesis contradicts other studies. However, there was also a lack of effect of acute exercise or regular training on salivary SIgA. Future studies should employ a more strenuous exercise bout (≥ 3 h) and/or conduct longitudinal monitoring of

athletes (e.g. elite) who have higher weekly training loads. These study designs would provide scope to elucidate any effect of COL on salivary SIgA and determine whether previously reported effects are not only limited to the type of supplement and/or methodology used.

5. Study 3 proposed the use of a novel *in vivo* marker of innate immunity (salivary bacterial load). Further research is required to investigate these findings in both a longitudinal and acute setting. It is expected that next generation sequencing of salivary bacteria would also provide a clearer indication of how salivary bacteria may be related to risk of URI.
6. Further study is required to determine why COL limited the increase observed in salivary bacteria load during the winter months. If salivary bacterial load does act as a risk factor for URI and the effects of COL purely reflect a blunting of seasonal variation only, then it is possible that COL may also be useful for the general population. It would be prudent to identify whether the effect of COL is due to maintaining immunity in response to factors (e.g. low Vitamin D status, He et al., 2013a) other than exercise training. Alternatively, more frequent analyses of salivary SIgA, sLac, sLys and other AMPs not yet considered (e.g. during the 'open window') in a training period may provide evidence to whether COL limits seasonal variation, acute changes in immunity or a combination of both.
7. It is becoming increasingly apparent that prior infection with latent herpesviruses (CMV, EBV) influences susceptibility to URI. Although there is conflicting evidence on the direction of this influence (positive or negative), it would be worthy to identify whether COL or any other nutritional countermeasure may modify such risk as reflected by possible preliminary evidence in study 4. Although there was no apparent role of EBV serostatus alone in risk of URI/URS

in Study 4, this thesis did not explore the incidence of EBV reactivations and/or its potential role as an *in vivo* marker of (acquired) immunity.

8. Following findings from study 5, further research is required to determine the relevance of the magnitude and/or the minimum threshold of contact hypersensitivity (response to DPCP) towards the risk of URI. Additionally, it would be prudent for investigators to perform concurrent analyses on other immune parameters during the induction and/or elicitation phase in order to determine where COL may be increasing the ‘sensitivity’ of the response or influencing host defence.

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Department of Sport and Exercise Science
Aberystwyth University
Health questionnaire (Updated 01/02/12)

STRICTLY CONFIDENTIAL

Please answer these questions truthfully and completely. The purpose of this questionnaire is to ensure that you are in a fit and healthy state to complete exercise associated with projects in the Department of Sport and Exercise Science.

1. How frequent would you describe your present level of vigorous activity?

   _____

2. Are you over 45 years (if male) or 55 years (if female)?
   Yes No

3. Do you suffer, or have you ever suffered from any form of heart complaint?
   Yes No

4. Do you have a family history of heart attack or sudden death below 65 years?
   Yes No

5. Do you suffer, or have you ever suffered from:
   - High Cholesterol Yes No
   - Asthma Yes No
   - Diabetes Yes No
   - Bronchitis Yes No
   - Epilepsy Yes No
   - High blood pressure Yes No

6. Do you smoke? Yes No

7. Have you had to consult your doctor in the last three months about any matter which may affect your ability to exercise? If yes, please give brief details

   _____

8. Are you currently taking any form of medication which may affect your ability to exercise? If yes, please give brief details

   _____

9. Do you currently have any form of muscle or joint injury?
   Yes No

10. Have you suffered from a bacterial or viral infection in the last two weeks?
    Yes No

11. Have you had cause to suspend your training in the last two weeks for a physical reason?
    Yes No

12. Is there any reason why you should not be able to successfully complete a test which requires a maximum effort?
    Yes No

13. If blood samples are being taken, do you suffer from shock symptoms, nausea or dizziness at the sight of needles or human blood?
    Yes No

Informed consent

The above information is correct to the best of my belief and I understand it will be treated in the strictest confidence. The experimenter has fully explained the purpose of the experiment and the possible risks involved. I understand that I may withdraw from the experiment at any time and that I am under no obligation to give reasons for withdrawal. I will adhere to the instructions of the experimenter regarding safety before, during, and after experimentation. I hereby volunteer to participate in experimental work as a participant.

Name of participant .....

Signature of participant .....

Date .....

Name of experimenter .....

Is supplementation part of these tests? Yes No

If yes, what? _____

Signature of experimenter .....

Date .....

Complete below if the experimenter is an undergraduate student.

No Health Issues declared ..... (Staff signature)

Health Issues Declared: Accepted? Yes No

Staff Name.....

Signature.....

SYMPTOMS OF ILLNESS AND GASTROINTESTINAL PROBLEMS

Your Name .....
Date .....

The following questions relate to any symptoms of illness and gastrointestinal problems **experienced today**. If a **symptom is present**, please place **a tick** in the box. If a symptom is not present, please leave blank.

If the symptom is **light**, please place an "**L**" in the box. If the symptom is **moderate**, please place an "**M**" in the box. If the symptom is **severe**, please place an "**S**" in the box.

Symptoms	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
	Tick if present	Symptom severity: Light (L), Moderate (M), Severe (S)	Tick if present	Symptom severity: Light (L), Moderate (M), Severe (S)	Tick if present	Symptom severity: Light (L), Moderate (M), Severe (S)	Tick if present	Symptom severity: Light (L), Moderate (M), Severe (S)	Tick if present	Symptom severity: Light (L), Moderate (M), Severe (S)	Tick if present	Symptom severity: Light (L), Moderate (M), Severe (S)	Tick if present	Symptom severity: Light (L), Moderate (M), Severe (S)
Fever														
Persistent muscle soreness or tenderness (more than 8 hours)														
Sore, painful throat														
Catarrh in the throat														
Runny nose														
Cough														
Repetitive sneezing														
Joint aches and pains														
Weakness/fatigue														
Headache														
Loss of sleep														
Inability to train/compete Light (L) = normal training maintained, Moderate (M) = training reduced, Severe (S) = training discontinued														
Loss of appetite														
Stomach upset														
Vomiting (being sick)														
Abdominal pain														
Diarrhoea														

SYMPTOMS OF ILLNESS AND GASTROINTESTINAL PROBLEMS

Your Name .....
Date .....

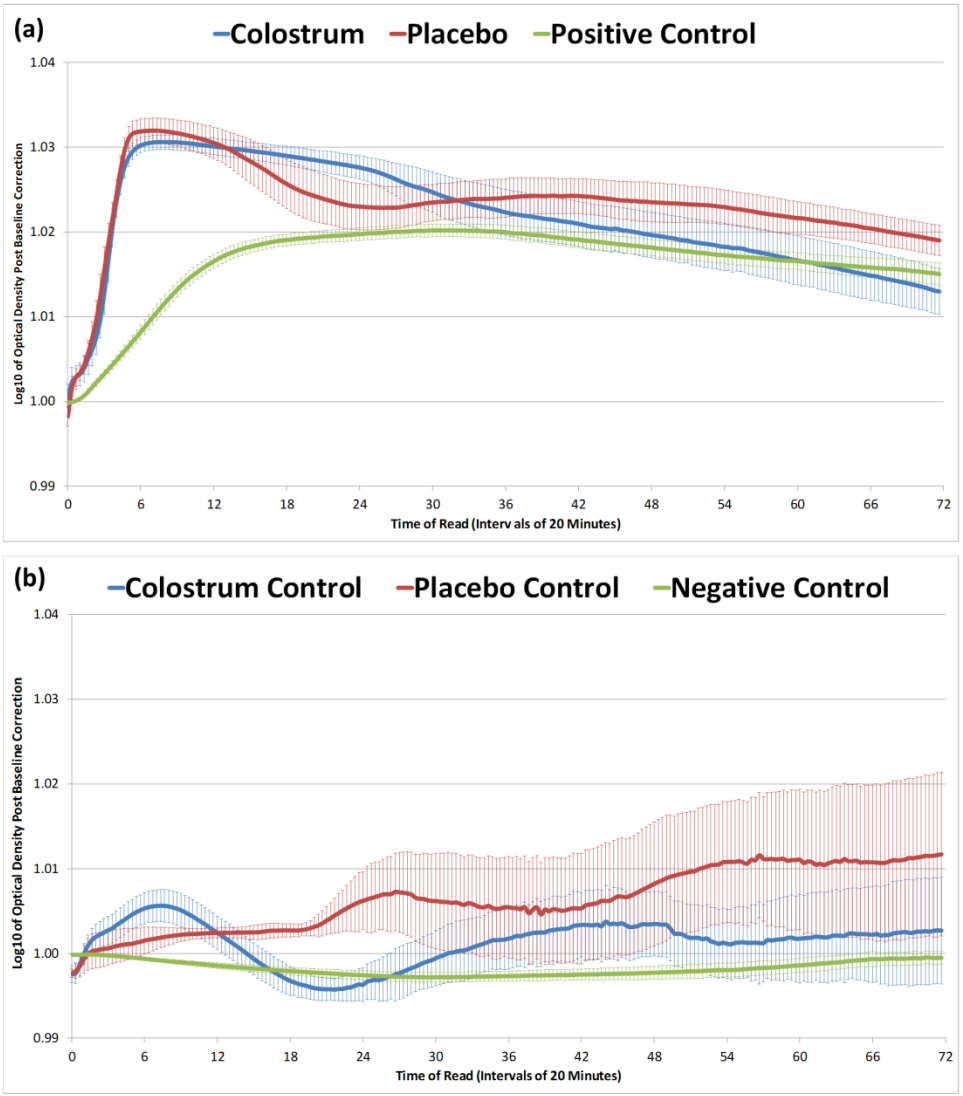
Have you taken your supplement? *(Please tick ✔)*	*Day 1*	*Day 2*	*Day 3*	*Day 4*	*Day 5*	*Day 6*	*Day 7*

a) Have you taken any medication this week? *Yes* ☐ *No* ☐ *If yes, please specify (i.e. type, what day)* .....

b) Have you visited your doctor/GP about any of the above symptoms this week? *Yes* ☐ *No* ☐

c) Has anyone who you have been in close contact with this week suffered from any of the listed symptoms? *Yes* ☐ *No* ☐ *If yes, please specify symptoms* .....

Appendix C



Microbial growth analysis shows no indication of a direct antimicrobial effect of either bovine colostrum (COL) or placebo (PLA) supplement (a), and also a level of microbial contamination in both the COL and PLA supplement (b).